The influence of novel poxvirus-based HIV-1 vaccine candidates on antigen presentation by dendritic cells

Von der Fakultät für Lebenswissenschaften

der Technischen Universität Carolo-Wilhelmina

zu Braunschweig

zur Erlangung des Grades eines

Doktors der Naturwissenschaften

(Dr. rer. nat.)

genehmigte

D i s s e r t a t i o n

von

Philip Rosenstock

aus

Bad Hersfeld

The influence of novel poxvirus-based HIV-1 vaccine candidates on antigen presentation by dendritic cells

Von der Fakultät für Lebenswissenschaften

der Technischen Universität Carolo-Wilhelmina

zu Braunschweig

zur Erlangung des Grades eines

Doktors der Naturwissenschaften

(Dr. rer. nat.)

genehmigte

D i s s e r t a t i o n

von Philip Rosenstock

aus Bad Hersfeld

1. Referent: Professor Dr. Stefan Dübel
2. Referent: Professor Dr. Ralf Wagner
eingereicht am: 24.01.2012
mündliche Prüfung (Disputation) am: 20.04.2012

Druckjahr 2012

Vorveröffentlichungen der Dissertation

Teilergebnisse aus dieser Arbeit wurden mit Genehmigung der Fakultät für Lebenswissenschaften, vertreten durch den Mentor der Arbeit, in folgenden Beiträgen vorab veröffentlicht:

Tagungsbeiträge:

Philip Rosenstock, Josef Köstler, Jens Wild, Kathrin Kindsmüller, Katharina Böckl and Ralf Wagner: HIV Vaccine Design to Improve Gag, Pol, Nef and Env Specific Immune Responses. (Poster) 21st Annual Meeting of the Society for Virology, Freiburg (2011).

Contents

A Summary

Cytotoxic T lymphocytes (CTL) directed against the HI-viral proteins Gag and Pol have been shown to control HIV replication efficiently. Thus, a vaccine eliciting CTL responses against conserved regions of Gag might induce protection.

Recently, in a clinical phase-I trial the immunogenicity of the vaccine candidate NYVAC-C was analyzed. NYVAC-C, consisting of the poxviral NYVAC vector coding for an artificial polyprotein Gag/Pol/Nef (GPN) as well as the envelope protein gp120 derived from the HIV-1 isolate 97CN54, was shown to induce only modest Gag- and Pol-specific CTL responses in few participants. The weak immunogenicity of NYVAC-C is believed to be evoked mainly by its low antigen expression rate. Thus, a new generation of immunogens based on GPN has been developed. By deletion of gp120, potential Env-induced suppression of Gag responses was avoided. To increase the steady-state levels of Gag, the natural ribosomal frameshift was reconstituted, resulting in expression of both, GPN and Gag. Additionally, size-reduced immunogen variants not containing Pol/Nef were designed. To further increase immunogenicity, antigen variants that allow formation of virus-like particles (VLPs) were developed. Again, due to its immunogenicity and excellent safety profile, NYVAC was chosen as antigen delivery system.

Since CTL activation requires efficient peptide presentation on antigen presenting cells (APC), in this work, the new generation vaccines were analyzed concerning their capacity to induce peptide presentation on APC in an *ex vivo* assay. Peptide presentation on APC can be elicited by either intracellular (direct presentation) or extracellular immunogens (cross presentation). As a model system, monocyte-derived dendritic cells (mdDC) were used as APCs. Antigen presentation was detected by restimulation of an HIV-1 Gag-specific CTL-clone that specifically recognizes the Gag derived peptide GL9.

First, to analyze the direct presentation capacity, mdDCs were infected with the vaccine candidates panel. As determined by restimulation of the GL9-specific CTL, immunogen-derived peptides were efficiently presented on mdDC despite incomplete mdDC maturation. Surprisingly, the weakly expressed antigen GPN induced most extensive direct presentation, whereas high expression of the new generation immunogens did not boost direct presentation.

Second, for observation of cross presentation, vaccine candidate infected HeLa cells were analyzed to induce GL9 presentation on mdDC. Although poorly expressed in HeLa cells, GPN and GPN in combination with gp120 induced the most extensive CTL restimulation by GL9-cross presentation on mdDC. Cross presentation of the new generation immunogens was induced only remotely and demanded high NYVAC multiplicities. It was shown that cross presentation was mainly driven by vaccine infected HeLa cells whereas Gag-derived VLPs did not induce GL9 presentation on mdDC. By using plasmid vectors as an alternative delivery system it was demonstrated that the unexpected low antigen presentation of the new generation antigens was not induced by the NYVAC vector itself.

For further analysis of peptide presentation, restimulation of Gag-p17-specific CTL was observed. Presentation of the SP9 peptide was not detectable indicating that not every single peptide encoded in an immunogen can be presented on an APC.

Thus, for induction of Gag specific CTL responses, the artificial polyprotein GPN appears to be appropriate. For better induction of CTLs in humans, more effective expression without affecting the immunogenic properties of GPN is required.

A.1 Zusammenfassung

HIV-1 Gag und Pol spezifische zytotoxische T Lymphozyten *(cytotoxic T lymphocytes*, CTLs) können die HIV-Replikation effizient unterdrücken. Eine wirksame Impfung erfordert die Induktion von CTLs, die gegen konservierte Regionen im Gag-Protein gerichtet sind.

In einer klinischen Phase-I Studie wurde die Immunogenität des Impfstoffes NYVAC-C, der für das künstliche Polyprotein Gag / Pol / Nef (GPN, Isolat 97CN54) sowie das Hüllprotein gp120 kodiert, untersucht. Der Impfstoff induzierte nur geringe Gag- und Pol-spezifische T-Zell Antworten in Studienteilnehmern. Es wird angenommen, dass die schwache Immunogenität der Antigene hauptsächlich durch deren geringe Expression verursacht wurde. Aus diesem Grund wurde eine neue Generation von Immunogenen basierend auf GPN entwickelt. Um die Gag-Expression zu erhöhen, wurde die ursprüngliche ribosomale Leserasterverschiebung wiederhergestellt, die zur Expression von Gag sowie GPN führt. Zusätzlich wurden verkürzte Immunogenvarianten ohne Pol/Nef entwickelt. Um die Immunogenität weiter zu verstärken, wurden Immunogene entwickelt, die die Fähigkeit besitzen, Virus-artige Partikel (*virus-like particles*, VLPs) zu bilden. Unter der Annahme, dass die Expression von Env zur Verringerung der Gag-spezifischen Immunantworten beiträgt, wurde gp120 in den neu-entwickelten Immunogenen nicht berücksichtigt. Zur Verabreichung der Immunogene wurde das gut verträgliche NYVAC-Vektorsystem verwendet.

Die Aktivierung von CTLs erfordert effiziente Peptidpräsentation durch Antigen-präsentierende Zellen (*antigen presenting cells*, APCs). In der vorliegenden Arbeit wurden die neu-entwickelten Impfstoffkandidaten auf ihre Fähigkeit zur Induktion der Peptidpräsentation durch APCs in einem *ex vivo* System analysiert. Die für die Aktivierung von CTL benötigte Peptidpräsentation kann entweder durch intrazelluläre (*direct presentation*) oder extrazelluläre Proteine (*cross presentation*) hervorgerufen werden. Als Modellsystem wurden aus Monozyten generierte dendritische Zellen (*monocyte-derived dendritic cells*, mdDC) verwendet. Vakzin-induzierte Antigenpräsentation wurde durch die Restimulierung von Gag-spezifischen CTL-Klonen bestimmt.

Um das Potential der Vakzinekandidaten zu untersuchen, *direct presentation* zu induzieren, wurden mdDC mit den verschiedenen Varianten infiziert. Trotz unvollständiger APC-Maturation wurde eine umfangreiche Präsentation des Gag-p24 spezifischen GL9-Peptides durch CTL-Restimulation festgestellt. Überraschenderweise induzierte das schwach exprimierte Ausgangsantigen GPN umfangreichere Peptidpräsentation als die stark exprimierten Immunogene der neuen Generation.

Des Weiteren wurden die Vakzinekandidaten hinsichtlich ihrer Fähigkeit analysiert, GL9 *cross presentation* auf mdDC auszulösen. Trotz geringer Expression in HeLa-Zellen induzierte GPN bzw. GPN in Kombination mit gp120 die umfangreichste CTL-Restimulation durch *cross presentation*. Die Immunogene der neuen Generation induzierten *cross presentation* in geringerem Umfang und nur unter Einsatz hoher Viruskonzentrationen. *Cross presentation* wurde hauptsächlich durch infizierte Zellen ausgelöst, VLPs induzierten keine Peptidpräsentation auf mdDC. Durch die Verwendung von Plasmidvektoren konnte gezeigt werden, dass die unerwartet niedrige Antigenpräsentation nicht durch den NYVAC-Vektor selbst hervorgerufen wurde.

Zur weiteren Untersuchung der Peptidpräsentation wurde die Restimulation eines Gag-p17 spezifischen CTL-Klons untersucht. Da dieses Peptid nicht auf mdDC präsentiert wird, konnte gezeigt werden, dass nicht jedes im Immunogen enthaltene Peptid effizient auf APCs präsentiert wird.

Obwohl das künstliche Polyprotein GPN die stärkste Antigenpräsentation auf APC hervorruft, erfordet eine effektive GPN-Vewendung in Impfstoffen eine Verstärkung der Expression, allerdings ohne die Immunogenität des Proteins zu verringern.

B Introduction

B.1 The Human Immunodeficieny Virus (HIV)

B.1.1 History and epidemiology

Since its discovery in the year 1983 [1], HIV distributed globally and became one of the major health challenges in poorly developed countries [2]. UNAIDS assumes that 33.3 million people were HIV infected by the end of the year 2009. It is estimated that in the last ten years an average of 2.5 million people became newly infected with HIV while 2 million AIDS related deaths occurred per year.

If left untreated, an HIV infection will lead to rapid depletion of $CD4^+$ T helper cells, thus promoting opportunistic infections and the development of tumors. The application of a highly active antiretroviral therapy (HAART) can diminish viral replication and extend the expectancy of life [3]. However, for global control of the viral spread, HAART is unsuitable because it (i) is poorly distributed in developing countries, (ii) leads to adverse side effects and (iii) cannot cure infection[4]. To decelerate or suppress virus spread, the combination of risk-reduction counseling, distribution of condoms in poorly developed countries and the development of an efficient vaccine appear to be best long-term perspectives [5].

B.1.2 Virus composition

HIV, a member of the retrovirus family, is a membrane surrounded lentivirus with a single stranded RNA genome. The characteristics of an HIV infection are a chronical course of disease and a long incubation period [6,7]. Since HIV is a highly diverse and variable virus, differences in the virus composition exist. Based on variations of the envelope protein (Env) the three major groups M (main), O (outlier), N (non-main or non-outlier) and P (pending the identification of further human cases)[8] could be identified. Group M is most widespread and subdivided into the nine clades A, B, C, D, F, G, H, J and K. Clade B viruses have the highest prevalence in Northern America and Europe, while viruses of Clade C which, at the moment, elicit the highest rate of new infections, are mainly found in Africa and Asia [9,10].

Virus particle composition and HIV replication have been reviewed extensively. For detailed description see the reference books [11,12,] as well as review papers [13,14]. In summary the HIV-1 particle is 100 – 140 nm in diameter and round or icosahedral (see Fig. B1). Since the viral membrane has emerged from the host cell, it carries numerous cellular components such as surface and transmembrane proteins, as well as the viral envelope proteins gp41 and gp120. Each particle incorporates seven to 15 gp120 trimers on its surface, which cluster at a distinct

region [15]. gp120 is attached to the membrane by non-covalent binding to the transmembrane protein gp41. The particle shape is determined by a lattice composed of matrix molecules (MA, p17). The p17 matrix contains a cone-shaped capsid comprising 1200 – 2000 capsid proteins (CA, p24), which is anchored on the matrix by p6. The capsid harbours two copies of the single stranded RNA genome as well as the viral proteins reverse transcriptase (RT, p66 / p51), nucleocapsid (NC, p7), integrase (IN, p32), protease (PR, p11), the viral regulatory protein negative regulatory factor (Nef) as well as the accessory proteins viral protein R (Vpr) and virion infectivity factor (Vif).

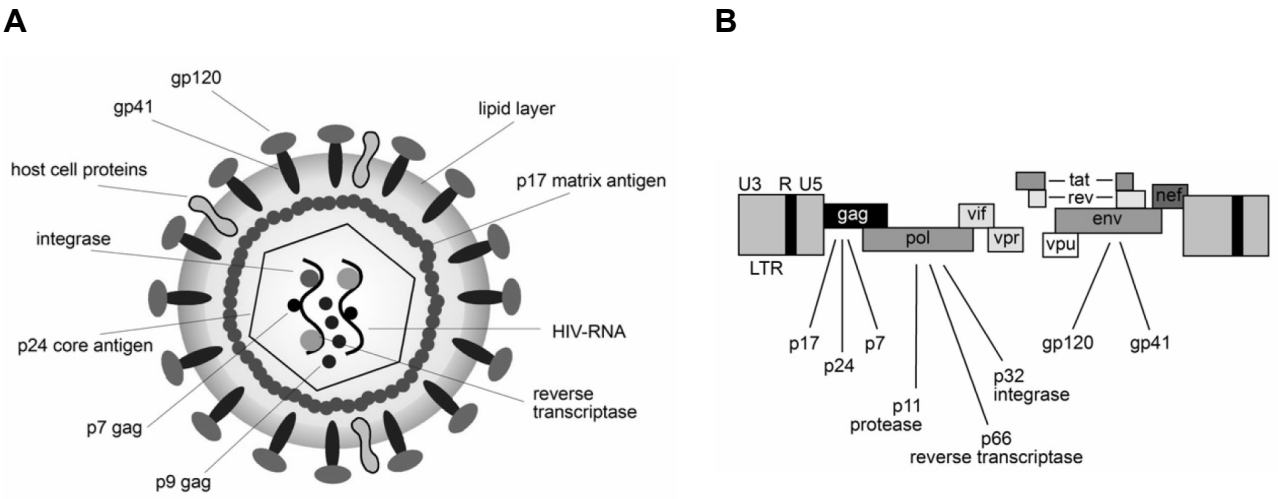


Fig. B.1 HIV-1 structure and genome organization [16]

A, Two copies of the ssRNA bound to p6 and p7 Gag as well as viral enzymes required for viral replication are surrounded by a conical capsid composed of p24 Gag. A matrix consisting of p17 Gag builds a lattice that forms the virus particle's shape. The host cell derived membrane harbours HIV envelope trimers and host cell proteins. *B*, Common to many retroviruses the HIV-1 genome's structural composition is 5' LTR-gag-pol-env-LTR 3'. Next to the major genes coding for structural proteins the genome contains several non-structural accessory and regulatory genes that are unique for HIV-1 (*vif*, *vpu*, *vpr*, *tat*, *rev* and *nef*)

Virus attachment to the cell is mediated by interactions of gp120 with the cellular CD4 surface receptor. CD4 is mainly expressed on cells belonging to the immune system such as T lymphocytes, macrophages, dendritic cells (Langerhans cells) and monocytes. The adhesion to CD4 leads to a conformational change of gp120 which in turn allows binding to either the coreceptor CCR5 or CXCR4. Rearrangements of gp41 result in a fusion of viral and host cell membrane. Towards uptake, the capsid is released to the cytoplasm in an uncoating process. Components embedded in the capsid are discharged into the cytoplasm of the host cell. Combined with MA, PR, NC, IN, and Vpr, the error prone RT forms a reverse transcription complex (RTC) which is responsible for the transcription of the RNA into dsDNA. Synthesis of the viral DNA genome as well as the CA dissociation induces the formation of the pre-integration complex (PIC) which guides the viral components to the cell nucleus. Due to signal sequences,

the PIC can enter the nucleus of non-dividing cells despite its size of 50 nm. The viral integrase inserts the HIV genome into transcriptionally active sections of the host cell's genome. Proviral genes are transcribed by the cellular RNA-polymerase-II enzyme complex. Early in infection, mRNA coding for the proteins transcriptional transactivator (Tat) as well as the regulator of virion (Rev) is synthesized, initiated at promoter regions in the 5' LTR. Tat enhances transcription rates by increasing RNA-Polmerase II activity. The interaction of Rev with the rev responsive element (RRE) located in the ORF coding for Env prevents the viral mRNA of being spliced completely and mediates the export of incompletely and unspliced mRNAs into the cytoplasm. Incompletely and unspliced mRNAs, coding for p55 Gag, GagPol, gp120 and gp41 as well as the precursor protein gp160, are translated at the rough ER. After trimerization, gp160 is transported to the Golgi complex and processed into gp41 and gp120 which are subsequently modified by glycosylation. In the cytoplasm the Gag precursor protein Pr55 that incorporates MA, CA, NC, p6, p2 and p1 as well as the GagPol protein precursor that comprises MA, CA, NC, p2, PR, RT and IN are synthesized. The GagPol coding mRNA is a template for the generation of both polyprotein precursors. A region located in the p1 and p6 ORF contains two conserved RNA elements, (i) a slippery site consisting of a UUUUUUA nucleotide sequence which leads to a reading frame switch and (ii) a stem loop region that causes a delay in the ribosomal motion. Together, both elements cause a -1 reading frame switch in approximately 5 % of the translation events which leads to the synthesis of the polyprotein precursors Gag and GagPol in a ratio of 20 : 1 [17,18]. To allow membrane anchoring, the different Gag and GagPol precursor molecules are post-translationally modified by myristoylation. Myristic acid, a saturated fatty acid named after the nutmeg *Myristica fragrans*, is transferred to the N-terminal Glycine residue by the enzyme N-myristoyltransferase [19,20].

For efficient particle formation the transport to the plasma membrane is mediated by interaction of p6 with the cellular ESCRT complexes, regularly located at endosomes. Together with Env, the myristoylated precursor proteins Gag and GagPol are anchored into lipid raft regions in the plasma membrane together with the immune regulatory protein Nef which promotes virus particle assembly [21,22]. For the formation of infective particles, two copies of the viral RNA genome are recruited by the interaction between a stem-loop-structure in the 5' UTR (Ψ-site) and NC. If the viral genome is not synthesized in adequate amounts or packaging is defective, non infectious *virus like particles* (VLP) lacking the viral genome will be formed [23]. For the generation of VLP, the Gag precursor protein is sufficient.

The final budding process proceeds simultaneously to the maturation process of the viral proteins. Excrescence of the plasma membrane is induced by formation of a p17 lattice structure. Activation of the immature viral protease (PR) is induced by dimerization of GagPol. PR transformes the GagPol precursor protein into its maturated functional subunits.

B.1.3 Vaccination

Underestimating the high variability of the virus, the US Secretary of Health Margaret Heckler stated in 1985, that a vaccine directed against HIV would be available within two years [24]. By now, more than 35 candidate vaccines were tested in about 150 clinical phase I/II studies without achieving satisfactory immune protection against an HIV infection[25]. In the phase III clinical trial RV 144 ("Thai trial") modest protection against infection was observed. Vaccination resulted in a 31 % reduced infection rate compared to placebo receiving persons. It was the first trial which showed effectiveness in preventing HIV infection [26,27].

The main obstacles in developing an effective HIV vaccine are (i) the lack of knowledge of immune correlates and protection, (ii) the difficulty in inducing broadly neutralizing antibodies due to virus variability and (iii) the absence of an animal model displaying similar disease and infection patterns.

When progressing, an HIV infection leads to the formation of quasi species [28] induced by selective pressure driven by components of the immune system such as cytotoxic T lymphocytes (CTL). Viral escape mutations induced by selective pressure of the immune system are advantaged by the high virus replication capacity [29], the host RNA-polymerase II, as the well as the error-prone viral enzyme reverse transcriptase [30]. Unlike other polymerases, a proofreading mechanism is missing, leading to an error rate of 10^{-3} to 10^{-4}. Together, those processes lead to continuous variation in the viral genome that corrupts elicitation of a protective antibody response.

B.1.3.1 Neutralizing antibodies directed against envelope proteins

First approaches of generating immunity against HI viral infection were based on the induction of neutralizing antibodies against the envelope protein. This development was unexpectedly hampered by extensive modulations in the gp120 structure. Large gp120 variations between different clades [31] as well as genetic recombination of different subtypes in an infected patient demand the elicitation of cross reactive antibodies which are hardly achievable [32,33]. Besides genetic variations, the accessibility of Env gp120 and gp41 epitopes is restricted by (i) N-glycosylation [34], (ii) conformational changes elicited by trimerization [35] and CD4 receptor binding [36]. Taken together, continuous evolution within one individual, as well as between different population groups and geographical regions represents a major challenge for the development of HIV vaccines based on broadly neutralizing antibodies [37,38].

When starting the development of an HIV vaccine, different classical vaccination strategies were explored. The approaches of administering (i) formalin or heat inactivated HIV-1 particles or (ii) Env-based subunit vaccines such as recombinant, soluble gp120 or gp140 (gp120 combined with the ectodomain of gp41 induced modest titers of antibodies that could not induce statistically significant reduction of HIV infection [39].

Current research in gaining broadly neutralizing antibodies is focusing on conserved epitopes located in the CD4 binding site of gp120 and masked regions in gp41, which both become accessible when binding to the host cell [40,41].

B.1.3.2 Generation of protective CTL responses

One of the most frequently discussed issues in development of a preventive HIV vaccine is whether it should induce sterilizing immunity rather than improved virus control after infection [42]. Due to HIV's persistent life cycle, sterilizing immunity can only be achieved by hindering virus entry into target cells [43]. $CD8^+$ CTLs are capable of controlling HIV replication, a fact that was first shown by the correlation between the appearance of virus specific CTLs and the decline of the primary viremia in acute infections [44,45]. CTL-deficient or anti-$CD8^+$ antibody treated macaques show high viremia and cannot control virus infection [46]. A stable, non-progressing HIV-1 infection in patients not generating efficient neutralizing antibodies, is usually induced by protective CTLs [47,48], directed mainly against HIV-1 Gag and Pol derived epitopes. Gambian and Kenyan seronegative sexworkers with high HIV-1 exposure were found to have HIV-1 specific protective CTLs [49].

Nevertheless, CTL driven sterilizing immunity is only detected in rare cases [50]. Cellular immune responses do not prevent the virus from generating a persistent infection. High plasma virus load can be observed despite high levels of HIV-1 specific CTL, mainly elicited by (i) escape mutations and (ii) ineffective T cell responses [51,52]. The effectivity of CTL responses is highly influenced by the patient's HLA-genotype (see B.2.2.3.2), which hampers the development of a universal vaccine mediating adequate protection independ of the HLA-type [53].

Thus, induction of highly protective CTLs cannot prevent seroconversion but can help to control virus replication after infection has occurred. Reduced viral plasma load suppresses disease progression associated with diminished transmission rates [54]. Despite all doubts concerning insufficient protection induced by T cell vaccination, the absence of a vaccine inducing broadly neutralizing antibodies demonstrates the necessity for the development of adequate T cell vaccines.

B.1.3.3 Viral vectors as delivery systems

In addition to naked DNA vaccines, the use of recombinant viral vectors displays the majority of recent studies to develop a CTL-stimulating HIV vaccine. The advantages in using viral vector systems are (i) high gene expression capacity, (ii) long lasting immunity, (iii) large transgene capacity, (iv) inexpensive manufacturing and (v) stimulation of the innate immunity [55]. The risk of (i) reversions, (ii) uncontrolled replication, (iii) insufficient attenuation, (iv) cytotoxicity and (v) alteration of the host genome are the main challenges, that have to be circumvented [56].

To date, a variety of viral vaccine candidates were developed. They are mainly based on attenuated pox- and adenoviruses, some of which already are in advanced stages of clinical trials [57]. The first developed live recombinant vectors were attenuated adenoviruses as well as modified vaccinia viruses such as (i) canarypox virus vector (ALVAC), (ii) Modified Vaccinia virus Ankara (MVA), and (iii) New York Vaccinia Virus (NYVAC) [26,27,58,59].

For opposed reasons, the two most prominent HIV-related studies are the phase II clinical trial V520 from Merck (STEP trial) as well as the phase III clinical trial RV144. While the RV144 trial was the first approach where modest protection against an HIV infection was detected [26], the V520 trial had to be stopped due to the lack of efficiency [60].

Adeno-based live recombinant vectors were shown in multiple studies to elicit promising immunity against HIV-derived proteins while having an excellent safety profile [61]. The V520 trial was based on the administration of three recombinant, nonreplicating, human adenovirus serotype 5-vectored vaccines (MRK-Ad5). The vectors contained either an HIV-1 Clade B derived Gag, Pol or Nef transgene. The dose-escalating, blind, placebo-controlled study included a three-dose homologous prime / boost regimen with different amounts of viral particles. Even though no serious vaccine-related side effects were monitored, the study was discontinued due to the lack of efficiency [62]. The MRKAd5-HIV gag/pol/nef vaccine did not lead to a decrease in viral plasma load in vaccinated individuals. HIV-1 infections occurred more often in vaccine than in placebo-treated patients with pre-existing adenovirus type 5 immunity [63]. It is discussed controversially whether (i) the formation of Ad5 immune complexes created an improved environment for HIV replication in T cells [64] or if (ii) an Ad5 based expansion of memory $CD4^+$ T cells with increased susceptibility for HIV infection induced enhanced infection rates in the MRK-Ad5 treated individuals [65].

In the clinical phase III study RV144 a 31 % reduced infection rate of vaccinees compared to placebo receiving persons could be observed [26,27]. This clinical study was based on a prime / boost regimen consisting of ALVAC®HIV vCP1521 (Sanofi Pasteur) and an AIDSVAX B/E (GSK) vaccine component [27]. ALVAC®HIV vCP1521 was engineered to induce expression of subtype E HIV-1 gp120 (92TH023) linked to the transmembrane anchoring portion of gp41 (strain LAI), as well as HIV-1 Gag and protease (LAI strain) [58]. The boosting agent AIDSVAX B/E was developed as a bivalent vaccine, consisting of a preparation of recombinant gp120 protein from two types of HIV (GNE8 and A244) [66]. In the final analysis, 51 vaccine recipients became infected compared to 74 placebo recipients. The efficacy result was considered to be statistically significant [26]. Taken together, the RV144 trial was the first study showing effectiveness in preventing HIV infection. Nevertheless, for an effective and extensive control of HIV infection the protectivity of AIDS vaccine candidates has to be substantially improved.

B.1.3.4 NYVAC as a viral vector system

Subunit vaccines based on poxviral vectors have been studied extensively. Due to their excellent safety profile and immunogenic properties, these vectors display a promising vaccine delivery system. Additionally, the viral genome allows a long-term antigen delivery.

Members of the poxvirus family have a large double stranded DNA genome (up to 220 kb) encoding several hundred proteins, each controlled by their own transcriptional promoter [67]. After fusion, the viral core is released into the cytoplasm where transcription of early genes occurs [68]. The early gene products are involved in viral DNA synthesis and expression of intermediate and late proteins such as structural proteins, enzymes, and transcription factors [69]. The cytoplasmic virus replication occurs at replication centers or viral factories, enclosed by the rough ER [70]. When incorporating viral DNA, immature viral (IV) particles accumulate. In a maturation process intracellular mature virions (MV) are formed. IMV represent the majority of infectious progeny and mostly remain within the cell until lysis. Some IMVs leave viral factories and become wrapped by a double layer of intracellular membrane derived from the early endosomes or trans-Golgi network to form intracellular enveloped virus (IEV) [71]. After fusion with the host cell membrane, IEV lose their outer membrane but remain attached to the cell as cell-associated enveloped virions (CEV). When detaching, they form extracellular enveloped virions (EV) [72].

Poxviruses were among the first viruses used as gene transfer vectors. Several different attenuated strains were utilized successfully as live vaccines against the smallpox-disease [73]. Poxviral vectors provide high stability and ease of manufacturing as well as administration. Due to their genome size, they have a large packing capacity [74]. The vectors were shown to induce both humoral and cellular immune responses against various transgens [75,76]. Due to complication when using *vaccinia viruses* (VACV) as immunization agents [77], attenuated vectors such as Modified Vaccinia Ankara (MVA) and New York Vaccinia Virus (NYVAC) with enhanced safety profiles were developed.

The highly attenuated NYVAC strain was derived from a plaque isolate of the Vaccinia Copenhagen strain (VACV-COP) by deletion of 18 ORFs (see C.1.4.1) affecting mainly host range, pathogenicity and virulence functions [78]. The resulting vector was shown to have limited replication capacity in human cell culture lines leading to significantly diminished production of infectious particles. The reduced replication is triggered by increased phosphorylation of the initiation factor eIF-2α, which leads to a translational block of several late gene products and consequently the abrogation of MV formation. A correlation between blocked synthesis of late gene products and induced apoptosis in HeLa and mdDC was examined [79,80,81].

Although highly attenuated, NYVAC provides a high degree of gene expression and strong induction of immune responses [82,81]. Infected DCs are shown to (i) maturate incompletely and (ii) suppress expression of late viral genes only partially [79,83]. The expression of viral immune regulators that mainly target cytokines, chemokines and TLR-signalling pathways [84] as well as the

incomplete maturation allow the virus to evade from antiviral host responses. However, NYVAC pulsed DC are successful in priming antigen specific CTL [85,86].

Immunization studies show, that MVA and NYVAC recombinants are capable of inducing cellular immune responses directed against HIV in mice as well as non human primates [76,87]. In humans, NYVAC-based HIV-1 vaccines were well tolerated and induced modest but multifunctional immune responses in most of the recipients in combination with DNA priming [85,88]. To date, no NYVAC vector has reached clinical phase III, nevertheless poxviral vectors are promising candidates as delivery vehicles of foreign proteins and therefore can be used as effective clinical vaccines [89].

B.2 T cell vaccine design

$CD4^+$ helper T cells as well as $CD8^+$ CTLs provide potent defences against virus infections and intracellular pathogens [90]. CTLs recognize infected or tumor cells by the binding of its T cell receptor (TCR) to pathogen or tumor derived peptides. TCRs recognize a single peptide bound to an MHC class-I molecules with high specificity. To enhance binding affinity of the TCR-pMHC complex, CD8-MHC contact as well as the interactions of co-receptors such as CTLA4 and CD28 with CD80 and CD86 on professional antigen presenting cells (APC) are required [91,92]. When attaching to target cells, CTLs induce programmed cell death by the release of cytotoxins such as perforin, granzymes and granulysin. After perforin-mediated induction of aqueous channels into the target cell's membrane, granzymes can enter the target cell. These serine proteases induce apoptosis by activating caspase cascades [93,94].

As described (see B1.3.2) $CD8^+$ CTLs are capable of controlling HIV replication [44,95]. CTL responses directed against HIV can be observed in long term non-progressors (LTNP) and elite controllers [47,48]. LTNPs are individuals that can control an HIV infection without an antiretroviral therapy. Therefore, vaccination strategies inducing HIV specific CTLs could represent a potent agent to control virus spread despite not mediating sterilizing immunity. Nevertheless, previous vaccination approaches failed to induce protection against an HIV infection. For this reason, improvement of the Gag immunogen design is strongly demanded to circumvent escape mutations and ineffective T cell responses.

B.2.1 The role of dendritic cells in T cell activation and HIV infection

B.2.1.1 General characteristics of dendritic cells

Dendritic cells (DC), the most potent antigen presenting cells APC, are a heterogeneous group of cells located in blood, tissues and lymphoid organs [96]. They are involved in the generation of both innate and acquired immune responses and are the only cells that are able to induce primary immune responses [97]. Immature DCs are capable of taking up and processing foreign antigens and organisms. Subsequently, antigen presenting, maturated DCs are able to stimulate B and T lymphocytes efficiently.

Dendritic cells are either of lymphoid or myeloid origin and can be diversified into different subsets by their anatomical distribution and cell surface markers [98]. Plasmacytoid DC (pDC) and myeloid DC (mDC) are derived from $CD34^+$ stem cells via the plasmacytoid or lymphoid pathway. In addition, DCs can be generated from blood monocytes (monocyte derived DCs, mdDC) or bone marrow precursors (see Tab. B.1) [99]. Because of the low DC abundance *in vivo*, mdDC generated from $CD14^+$ monocytes stimulated with IL-4 and GM-CSF, are often utilized as a model system [99]. They offer features similar to mDCs but do not fully mimic the immunological functions of *in vivo* cells [100].

For the disposition to take up antigens, DC precursors have to be transformed into an immature state induced by the cytokines GM-CSF, IL-3 and IL-4 [101]. Immature pDC and mDC are present in blood and represent 0.2 – 1% of all PBMC. Langerhans-type DC (LDC), a subset of mDC, are homing to epithelial surfaces where they rest in an immature state in a concentration of 0.5 –2% until encountering antigen [98].

Tab. B.1 Characteristics and properties of different dendritic cells subsets

Name	Description and properties
Myeloid dendritic cell (mDC)	Morphology: similar to monocytes Functions: Major stimulator of T cells, some subsets are involved in wound healing
Langerhans cells (LDC)	Properties: subset of mDC, present in epidermis Functions: uptake and processing of microbial antigens that crossed the skin barrier
Plasmacytoid dendritic cell (pDC)	Morphology: similar to plasma cells Functions: activation of innate immune responses by interferon type I expression
Monocyte derived dendritic cell (mdDC)	Morphology and functions: similar to mDC Often utilized in research

Resident immature DCs possess a highly active endocytic system that is coordinated for efficient antigen processing. Several forms of antigen uptake are described for immature DC, such as i) macropinocytosis [102], ii) receptor-mediated endocytosis via C-type lectins [103], and iii) phagocytosis of necrotic or apoptotic cell fragments [104], viruses and bacteria [105]. Besides elevated antigen capture by endocytosis and phagocytosis, characteristics of immature DCs are secretion of the cytokines IFN-α, TNF and IL-1.

Stimuli such as bacterial lipopolysaccharide, tumor necrosis factor family ligands (TNF, CD40L, FasL), bacterial DNA or viral dsRNA as well as DNA can activate DCs [106]. These stimulants interact with Toll like receptors (TLRs) present intracellularly or at the cell surface of APCs. TLRs activate a signal cascade that initiates maturation of DC. Maturated DCs express a large spectrum of chemokines such as MIP-1α, MIP-3α and RANTES to attract immature DC [107]. The formation of a chemokine gradient leads to rapid DC accumulation at the sites of antigen presence within only one hour [108]. Additionally, in maturated DCs molecules necessary for activation of T cells such as MHC class-I, and the co-receptors CD80, CD86 are upregulated. Maturation is linked to DC migration from skin to secondary lymph nodes, driven by the chemokine receptors CCR7 and CCR6 [109] that mediate the escape from local MIP-3 gradients. Skin migration is facilitated by downregulation of the adhesion molecule E-cadherin [110], cytoskeleton remodelling and distribution of collagenase [111].

After accessing the draining lymph node, mature DCs are driven to the paracortical area where they spread all over and form a pervasive network in T cell areas [112]. To direct Ag-bearing DCs and naïve T lymphocytes into the paracortical area, resident mature DCs express cytokines such as MIP-1β [113]. In the lymph node, Ag-bearing DCs encounter naïve $CD4^+$ and $CD8^+$ cells. For efficient $CD8^+$ CTL stimulation, complete DC maturation is usually required. Therefore, $CD4^+$ T helper cells specifically enhance DC maturation mediated by CD40 ligand [114] that allows productive CTL priming.

B.2.1.2 Pathways of antigen processing and presentation in dendritic cells

For the activation of specific CTLs in lymph nodes, DCs have to present peptides derived from various antigens. For peptide presentation, antigens present in the cytoplasm or collected from the extracellular space are processed by the proteasome into small peptides that are translocated by the transporter associated with antigen processing (TAP) complex into the endoplasmatic reticulum (see B.2.1.3.2). There they form complexes with MHC class-I molecules (pMHC) which are presented at the surface of DC.

Proteasomeal protein processing in the cytoplasm is an essential cellular feature[115]. It plays a role in (i) degradation of misfolded, unfolded, damaged or mutated proteins [116,117], (ii) control of cell cycle and metabolic pathways [118], and (iii) recovery of amino acids maintained from the degradation of extracellular proteins. Intracellular antigens are degraded by an endogenous

proteolytic or lysosomal pathway, transported into the ER and loaded to MHC class I complexes. This process is called **direct presentation** [119,120]. Direct presentation can result in efficient CTL stimulation (see Fig. B.2) and therefore is involved in controlling virus infections [121].

Extracellular proteins, taken up by surface mannose receptors or C-type lectin receptors such as DC LAMP [122,123] are endocytosed and degraded via a lysosomal pathway by cathepsin proteases. Regularly, peptides derived from extracellular proteins are not targeted to MHC class-I compartments but are presented on MHC class-II molecules, that can activate $CD4^+$ lymphocytes rather than CTL [124,125]. Additionally, these peptides can be **cross presented** (see Fig. B.2) on MHC class-I molecules [126,127]. These observations have lead to the conclusion that some DCs have an alternative pathway to present peptides derived from extracellular antigens [128,129]. This pathway is believed to be necessary for the generation of immune responses against tumors [130] and pathogens, which do not infect APC [131]. Besides the generation of immune responses against exogenous antigens [122,132], cross presentation is also operative in the development of immune tolerance [133].

Two possible routes are discussed for peptide generation in the cross presentation pathway. In the Tap-independent process the antigen is hydrolyzed in endosomes [134], in the Tap-dependent phagosome-to-cytosol-pathway, the antigen is processed in the cytoplasm [135].

Cross presentation was described to be mainly driven by phagocytosis of cell bodies derived from apoptotic cells [136,137]. mdDC loaded with apoptotic bodies of influenza infected HeLa cells or DC are capable of stimulating specific CTL [104,138]. Nevertheless, structural mechanisms involved in cross priming are not examined accurately. Mainly, research focused on the impact of the antigen itself for cross presentation. It was shown that the structure and amino acid sequence of peptide precursor proteins affect cross presentation [139], at which sequence and length of the peptide flanking regions have the strongest influence[140]. Additionally, intramolecular localization of a given peptide in a protein determines the ability to be cross presented.

It was reported, that cross presentation is an important mechanism to induce immunity against viral pathogens and tumors [141]. Since direct and cross presentation cannot be separated *in vivo*, it remains unclear which process has most extensive impact on CTL priming. Although efficient CTL responses were described to be elicited by both direct and cross presentation alone, it is likely that both mechanisms interact in stimulating $CD8^+$ T lymphocytes. Certainly, the pathogen itself dictates whether direct or cross presentation is demanded for CTL activation. Stimulation of T lymphocytes directed against poxviruses was found to require cross presentation [142].

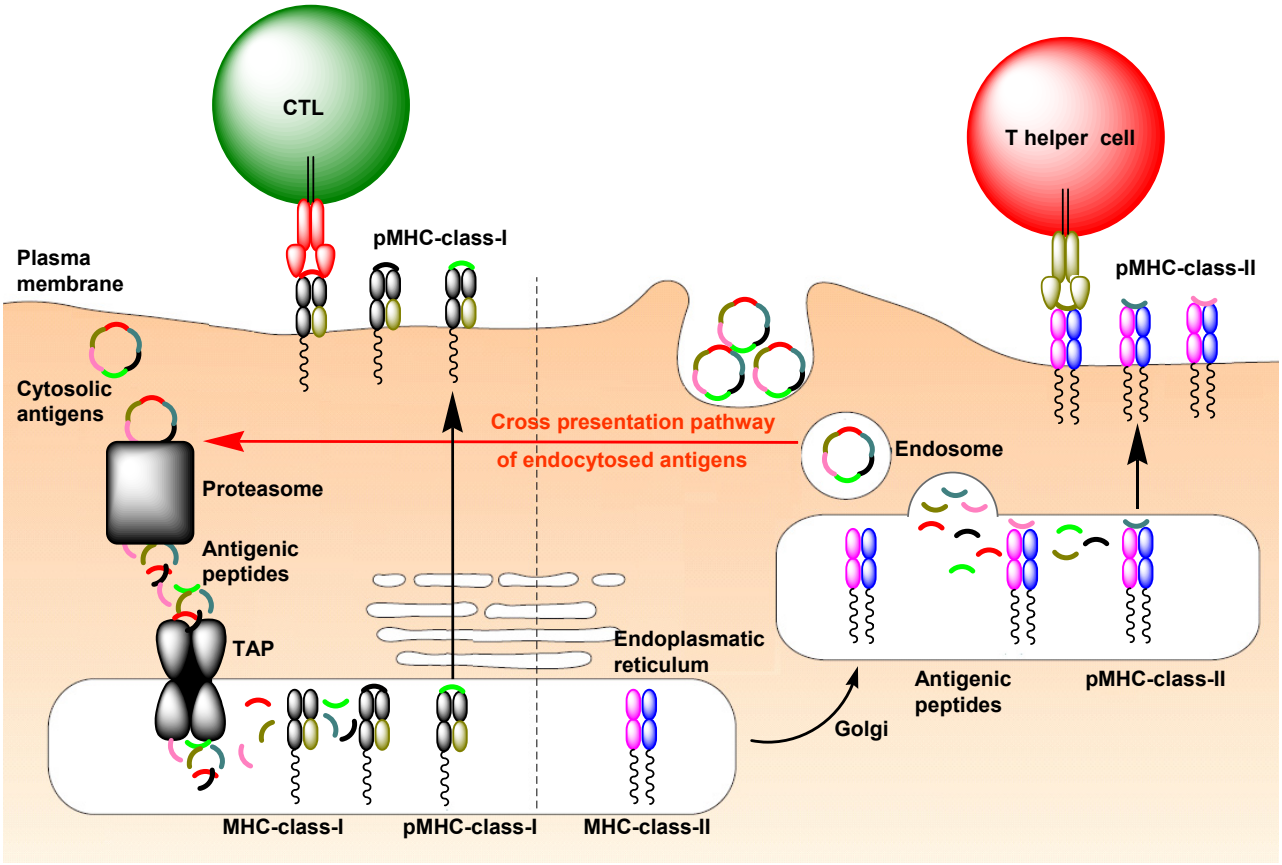


Fig. B.2 Peptide presentation by MHC class-I molecules can be induced by direct presentation of intracellular antigens as well as cross presentation of endocytosed antigens.

Generally, MHC class-I molecules present peptides that are derived by proteasomal digestion of cytosolic antigens. By the TAP complex these peptides are translocated into the ER where they are bound to MHC class-I molecules. After being located in the outer membrane, pMHC class-I complexes can activate $CD8^+$ T cells. In contrast, endocytosed antigens are associated with MHC class-II molecules. When being presented on the cell surface pMHC class-II molecules can activate $CD4^+$ T helper cells. In an alternative cross presentation pathway, endocytosed antigens can enter the route mainly found for cytosolic antigens. After proteasomal digestion and ER translocation, the peptides are loaded on MHC class-I molecules that can activate $CD8^+$ T cells (adapted from Heath and Carbone, Nature Revues, 2001 [141])

B.2.1.3 Mechanisms of antigen processing and presentation

B.2.1.3.1 Generation of antigen-derived peptides by proteasomeal digestion

To be accessible for presentation, antigens have to be processed proteasomealy into peptides. Proteasomeal digestion of antigens is directly influenced by its (i) amino acid sequence and (ii) immunogenicity. Template dependent variations in ubiquitination and proteasome digestion result in different cleavage rates and pattern. Immunogenic antigens initiating innate immune responses can alter proteasome composition (“immunoproteasome”) and therefore influence digestion.

For the generation of peptides, proteins located in the cytoplasm are processed in a proteolytic pathway requiring ubiquitination [117]. For ubiquitination of a protein substrate, a ubiquitin sidechain is conjugated to a lysine's amino group by an ubiquitination-complex [117,143]. The target protein's amino acid sequence as well as the character of ubiquitination strongly influences the modification process. Large, bulky and charged N-terminal amino acid residues lead to more rapid degradation rates [144] and therefore effective MHC class-I presentation [145,146]. By different compositions of ubiquitination-complexes, cells can selectively degrade proteins [117,147]. Targeted degradation is considered to be necessary especially for regulatory molecules such as NFκB, that controls multiple immune responses, cell proliferation and apoptosis [118].

Once being modified by ubiquitination, proteins are guided to cytosolic proteasomes for degradation. A number of studies indicate that the proteasome is a "multicatalytic protease" that exhibits different distinct protease activities that are driven by different subunits. The 20S core particle is able to process proteins after (i) basic, (ii) acidic, (iii) neutral, and after (iv) branched amino acids [148,149150]. When entering the proteasome, the residues P1, P4 and P5 adjust the peptide in the active site of the proteasome, which influences processing [151].

Protein proteolysis can by enhanced by (i) so called degron sequences [152] (ii) regions rich in prolin, glutamine, serine and threonine (PEST sequences) [153], as well as (iii) proline residues in peptide flanking regions [154,155].

Furthermore, IFN-γ strongly influences the proteasome composition. Immunoproteasomes possess altered substrate properties as well as an enhanced cleavage rate. Thus, changed catalytic properties affect protein digestion strongly. The proteasome's capacity to cleave peptides after hydrophobic residues is strongly increased whereas cleavage after acidic residues in reduced [156,157]. Thus, due to varying peptide generation the presence of immunoproteasomes might induce in altered peptide presentation resulting in activation of alternative CTLs.

B.2.1.3.2 Peptide translocation into the ER and formation of pMHC complexes

For presentation on the cell surface, processed peptides are translocated from the cytoplasm to the endoplasmatic reticulum by the transporter associated with antigen processing (TAP). The TAP transporter mediates signal peptide independent translocation of peptides from the cytoplasm into the lumen of the endoplasmatic reticulum [158,159]. Translocation of nonamers possessing hydrophobic C-termini is favoured by the TAP transporter [160]. Amino acid exchanges at positions 2 to 7 rarely affect the efficiency of peptide transport whereas proline present at position 3 strongly improves TAP translocation [161].

After the peptides have been transported into the ER, the assembly of *peptide MHC complexes* (pMHC) occurs [159]. pMHC complexes are capable of presenting proteasomeal derived peptides to $CD8^+$ T cells, thereby initiating CTL responses. The MHC heterodimer, composed of a membrane anchored-heavy α-chain, as well as the soluble β_2-micrglobulin, is an unstable complex that is

stabilized upon binding an adequate peptide [162]. The α-chain forms an interchain dimer, resulting in a peptide binding cleft [163]. The genes encoding the human MHC class-I components, the human leucocytes antigens HLA-A, -B and –C, are located in one of the most polymorphic mammalian genetic loci [164]. The allelic polymorphism strongly influences amino acids that determine specificity for foreign antigens [165,166]. Most highly variable residues are found to point into the binding groove conferring unique peptide- and TCR-binding properties on each MHC molecule [167,168]. The MHC complex binds peptides specifically, which strongly limits the number of possible binding peptides [169]. Nevertheless, bound peptides vary in sequence and length although the MHC-I binding cleft is sterically blocked at both sites [170]. This allows for the presentation of a large number of peptides in a limited number of MHC molecules. High diversity of peptide binding ensures resistance against a wide variety of pathogens, limiting immune evolvement and increasing breadth of peptide recognition [171]. This flexibility is achieved by the structurally equivalent binding of only a small number of each peptide's residues [172,173].

Binding motifs differ between different HLA alleles and are mostly influenced by character and number of contact sites [174]. These sites are determined by individual pockets in the binding grooves of different HLA molecules, which leads to interaction with distinct amino acids present at defined positions of the peptide [175]. Specific anchor positions vary over HLA alleles, but P2 and P9 as well as the co-anchor P3 are mainly found to influence binding [176].

Binding to the TCR stabilizes the pMHC complex by multiple contact sites. Different TCRs are found to contact different sites of the peptide and MHC side chains [177,178]. Although large parts are buried in the MHC binding groove, bound peptides are exhibited into the TCR recognition surface [179]. pMHC:TCR binding induces stimulation of a naïve CD8+ T lymphocytes or restimulation of activated CTL.

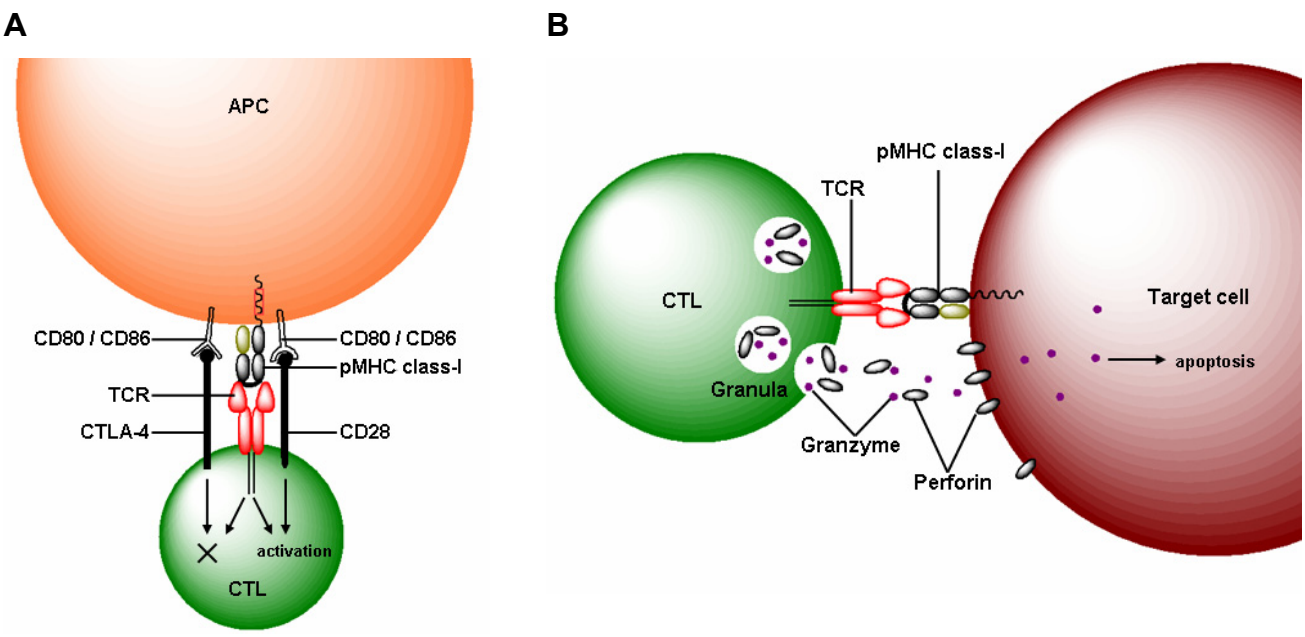


Fig. D.3 Activation and effector function of CTLs

A, CTL activation is mediated specific recognition TCR:pMHC class-I interactions. The TCR binds to an adequate immunogen-derived peptide as well as the MHC complex itself. Efficient activation requires interactions between co-stimulatory molecules such as CD80 or CD86 with CD28. When interacting with CTLA-4, an inhibitory signal is transmitted that suppresses CTL activation. B, Infected cells can display pathogen derived peptides by pMHC complexes. When an adequate CTL encounters the infected cell, contact between both cells mediated by specific TCR:pMHC class-I interactions. The contact induces activation of CTL resulting in degranulation and release of perforin and granyzme. When inserting in the plasma membrane perforin forms pores that lead to non-specific cell lysis. Additionally, these pores allow granzymes to enter the target cells. Granzymes are serine proteases that induce apoptosis by activating caspases.

B.2.1.4 The role of DCs in HIV infection

By inducing adaptive immune responses through secretion of type-I IFN [180], as well as activation of B and T lymphocytes, DCs play a major role in the defence against viral infections. Certainly, the uptake of HIV leads to modulations in the immune stimulation capacity of DC. In HIV infection, DCs are not only found to have favourable effects on disease progression but next to the initiation of antiviral immune responses, DCs also are associated with disease progression [181].

Accounting for approximately 80 % of new infections, heterosexual transmission is the main route of HIV-1 transfer. Because they are present at the oral and vaginal mucosa, DCs are among the first cells that encounter HIV [182]. HIV can enter DCs by different pathways. In some DC populations such as mdDC, HIV is taken up by receptor-mediated endocytosis. The viral entry is mediated mainly by lectins such as DC-SIGN [183] and macropinocytosis. Some subsets express small amounts of CD4 as well as the coreceptors CXCR4 and CCR5. Therefore, DC-fractions are susceptible to HIV infection [184]. Due to (i) low level receptor and coreceptor expression (ii) rapid degradation of internalized HIV, and (iii) host defence mechanisms only 1 - 3% of the total DC populations are infected productively [185]. Despite the low numbers of infected cells and production

of virus low amounts [186] DC are jointly responsible for the spread of HIV [187]. When interacting with T lymphocytes in the lymph node, a viral synapse allowing virus transfer between infected DC and lymphocytes is formed [188,189]. Trans-infection of $CD4^+$ cells mediated by DC-derived exosomes transmit HIV to T cells without *de novo* infection [190,191].

Due to reduced maturation, infected DC are less efficient in stimulating T cells [192,193]. Nef driven reduction in MHC levels as well as decreased levels of the corecepetors CD80 and CD86 impair but do not subvert T cell priming [194,195]. Secondly, cytokine productions are altered in HIV infected DC, leading to less protective immune responses [196].

It was shown that disease progression can be impaired by the patient's HLA genotype [197]. Three single nucleotide polymorphisms (SNP) that map close to the HLA loci are found to be associated with low levels of viremia during the asymptomatic phase of infection [198,199]. The International Controllers Study analyzed the HLA type of an infected cohort [200]. It was found that the HLA types B*57, B*27 and B*14 can be associated with protectivity against disease progression, whereas B*35 and C*07 are associated with a rapid progressor status [197,201]. Two possible rationals for the protectivity of distinct HLA molecules are discussed. First, it was found that protective HLA molecules bind fewer peptides and delete fewer self-reactive T cells in the thymus giving HIV-specific CTL broader fine specificity [202]. Thus, broader CTL responses reduce viral options for generating escape mutations [203,204]. Secondly, protectivity could be mediated by binding of conserved regions of HIV-1 Gag [205].

B.2.2 The role of CTL in HIV infection and vaccination

B.2.2.1 The function and malfunction of CTL in HIV infection control

CTLs are one of the key players of HIV control in early acute infection. The correlation between the appearance of virus specific CTL and the decline of primary viremia in acute infection demonstrate the influence of T lymphocytes on HIV replication [44,45]. In acute HIV infection virus spread can be controlled by to CTL responses whereas in advanced disease stages virus replication can no longer be suppressed due to (i) viral escape mutations and (ii) a switch in CTL population composition [44,206,207]. Additionally, the primary CTL response is directed against epitopes best accessible, which often do not mediate protection [208].

Beside escape mutations, HIV strongly influences CTL population composition to escape immune pressure. Disease progression is not associated with the numerical loss of CTL whereas the number of $CD4^+$ T helper cells is strongly decreased. In the early chronic phase, the amount of HIV specific CTLs in progressors is not diminished compared to LTNP [45]. It was observed, that a skewing in $CD8^+$ T cell differentiation leads to less functional CTL [209,210]. Although recruited during early infection, high avidity HIV-1 specific $CD8^+$ T cells subsequently get lost in the presence of persistent high-level viral replication due to a switch in clonotypic composition [211]. The inefficiency

of CTL responses to HIV is enhanced by suppression of polyfunctional memory $CD8^+$ CTL expressing Interleukin-2 (IL-2) and Interferon-γ (IFN-γ) [212]. Monofunctional IFN-γ expressing effector cells have reduced efficiency to decelerate or inhibit disease progression. HAART treated individuals show high levels of IL-2 expressing HIV-specific memory T cells whose numbers decline when starting a *structured treatment interruption* (STI) [213]. Beside the population composition switch upon entering chronic infection status, HIV leads to apoptosis of T lymphocytes in late chronic infection stages. The increased susceptibility to apoptosis of $CD8^+$ cells correlates with disease progression [214].

Even though present in all HIV infected individuals, the effectivity of CTL responses differs strongly. In patients with low or remote disease progression, a correlation between the HLA-genotype and protectivity of CTL epitopes was observed. Therefore, to arrest HIV infection in early stages with low viral plasma loads, an effective CTL vaccine has to suppress the development of unprotective CTL as well as escape mutants.

B.2.2.2 Composition of a potent HIV-1 antigen for CTL activation

For the induction of protective CTL that decelerate or suppress viral infection, initial $CD8^+$ T cell responses which have greatest impact on reducing viremia have to be intensified and stabilized (see B.2.2.1) [206]. The strongest immune responses induced in early infection are directed against early regulatory and accessory proteins such as Tat, Vpr and Nef as well as the structural protein Gag [215,216,217]. Due to their high mutation rates, accessory and regulatory proteins only play a minor role in the development of vaccines.

For induction of multiepitopic immune responses, a combined administration of different viral proteins could improve a vaccine's immunogenicity. Nevertheless, to date vaccination development mainly focuses on inducing Gag responses because of its variety of possible epitopes and conserved regions. Additionally, epitope enrichment within an antigen could improve its immunogenicity [218,219,220]. On the one hand, epitope enrichment might circumvent the presentation of just a limited number of eptitopes and lead to the development of generalized vaccines, which could be administered HLA-type independently. On the other hand, a variety of factors influencing peptide processing and presentation (see B.2.1.3), as well as the high HIV-1 sequence variability (see B.1.3) have to be considered. Secondly, most epitopes have been found to be clustering at distinct Gag regions which can impair coverage of all possible epitopes within one antigen [221]. Furthermore, it was revealed that some epitopes - although accessible for presentation - were not presented to T lymphocytes. Presentation of subdominant epitopes that could confer protection is inhibited frequently by immunodominant peptides [222]. In C57BL/6 mice it was shown, that the hierarchy of D^d CTL epitopes is clearly fixed. For the Lymphocytic Choriomeningitis virus (LCMV) model, only the three rarely presented epitopes GP-33, NPD-396 and GP-276 are responsible for eliciting specific CTL responses [223]. In HIV-1 infection,

crystallographic data suggested that only one peptide can adequately bind to HLA-B*2705, whereas no well-defined HLA-A*1 restricted epitopes has been described [224,225].

Even though escape mutations complicate the development of effective immunogens, induction of broadly cross reactive CTLs could circumvent this obstacle [226,227]. The HLA-A*2 restricted Gag-p17 epitope SL9 (SLYNTVATL, residues 77-85) is the best defined and examined HIV-1 epitope [228,229]. It was shown, that the immunodominant SL9 pepitde is found in approximately 70% of all HLA-A*2 patients in acute HIV infection whereas in chronic infection only a small part of patients presented this epitope indicating escape mutations [230].

To enhance immunogenicity, antigens capable of inducing cross priming of CTL are preferably employed as a vaccine. Especially when using vector systems that do not directly affect professional APCs, such as poxviral vaccines [142,231], antigens designed for enhanced cross priming capacity are demanded. Besides the protein half-life and amino acid sequence (see B.2.1.3), the accessibility of extracellular antigen is strongly facilitating cross presentation. The ability of Gag to form virus like particles (VLP) leads to elevated amounts of HIV-1 proteins being presented extracellularly. VLPs represent a form of subunit vaccines that are able to self assemble into an organized particle structure [232,233]. Due to the lack of regulatory proteins as well as genetic material, VLPs are both replication and infection incompetent. VLPs can efficiently induce cross presentation leading to MHC-class-I presentation of HI-viral antigens [234,235]. For VLP uptake, various mechanisms such as phagocytosis, clathrin-mediated uptake, non-clathrin-mediated uptake and macropinocytosis were described [236]. Most VLP models have been shown to induce activation and maturation of DC through TLR2 and TLR4 [237,238]. In animal models, VLPs were shown to induce both mucosal and systemic immune responses [239]. Especially when used in prime / boost regimens, VLPs induce cellular and humoral immune responses in mice [240,241].

B.2.2.3 The clinical phase-I trials EuroVacc01 and EuroVacc02

For the development of a safe and effective HIV-1 vaccine, novel polyepitopic antigens were engineered and analyzed by the EuroVacc foundation [85,88,242]. The most promising vaccine candidate was designed as a bivalent vaccine consisting of both the artificial polyprotein Gag/Pol/Nef (GPN) and gp120 based on the C-clade 97CN54 provirus isolate collected in Sianking province (China) [243]. For safety issues, several modifications were introduced such as (i) abrogation of VLP formation by interrupting the Gag myristoylation signal (G2A), (ii) inactivation of the protease (D577N), (iii) an exchange of C- and N-terminal regions in the *nef* gene sequence (nucleotides 8,170–8,469 linked to 3′ end of nucleotides 8,470–8,787), as well as (iv) interruption of the Pol ORF by inserting the scrambled nef sequence at position 2,406 – 2,527 (see C.1.3). Due to an excellent safety profile and immunogenic properties of the vector, the GPN and gp120 gene sequences were inserted into TK locus of NYVAC (NYVAC-C). Transgene expression was

driven by a bidirectional NYVAC *early / late* (E/L) promoter. Thus, gene expression leads to equal amounts of GPN and gp120.

In the two clinical phase-I trials EuroVacc01 and EuroVacc02 (EV01 and EV02), immunogenicity and safety issues of the polyepitopic C-clade antigen were evaluated [85,242]. No serious adverse events were monitored in the placebo-controlled phase-I study EV01 [242]. In the EV02 study, the administration of NYVAC-C resulted in modest cellular immune responses in some patients mainly directed against HIV-1 gp120, whereas Gag, Pol and Nef specific responses were detected to be weak (see Fig. D.4). Since (i) protective CTL epitopes were found to be mainly located in Gag and Pol and (ii) Gag comprises several highly conserved regions, a vaccine that induces Gag-specific CTL is believed to mediate protection [215,216,217]. Thus, NYVAC-C did not fulfil the demanded vaccine characteristics.

Thus, for expanding breadth and depth of the Gag specific immune responses a new generation of immunogens was developed (see D.1). Based on the results of the EuroVacc studies it was estimated that gp120 negatively influences Gag-specific CTL responses. Additionally it was assumed, that GPN size reduces expression strongly. Thus, the new generation immunogens were considered to (i) be expressed more efficiently, (ii) increase cross presentation, and (iii) avoid replacement of Gag epitopes by immunodominant Env, Pol or Nef peptides (see D.1).

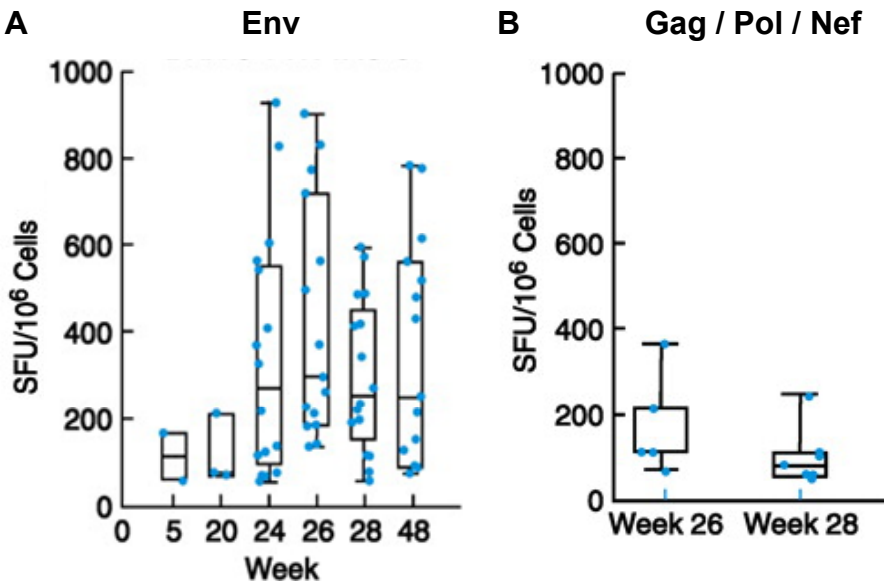


Fig. D.4 HIV-specific T cell responses detected in PBMC of participants of the clinical phase-I trials EV02

Human volunteers were inoculated with DNA-C (week 0 and 4) and boostered with NYVAC-C (week 20 and 24). PBMC were isolated, restimulated and analyzed in an IFN-γ ELISPOT assay. (A) Median magnitude of Env-specific T cell responses at different time points across the duration of the study; (B) Median magnitude of Gag-, Pol-, and Nef-specific T cell responses at weeks 26 and 28. [88].

B.2.3 Objective of the present study

Gag-specific cytotoxic T lymphocytes (CTL) play a major role in HIV infection control. Thus, vaccination requires efficient induction of Gag-specific CTL. For this purpose, the vaccine candidate NYVAC-C has been developed by the EuroVacc foundation. In the clinical phase-I study EuroVacc02 the vaccine candidate NYVAC-C failed to induce extensive T cell responses which was most likely provoked by modest immunogen expression. On this account, NYVAC-based vaccine candidates with newly designed immunogens, based on the initial GPN polyprotein that was applied in NYVAC-C, were developed. Since stimulation of CTL was believed to require efficient antigen levels, the immunogens were designed to be extensively expressed. To increase the steady-state levels of Gag, the natural occurring ribosomal frameshift was reconstituted resulting in expression of both Gag and GPN in a ratio of 95:5. Additionally, to reduce the immunogens' size further, several immunogen variants not containing Pol/Nef were developed. Since extracellular proteins can induce cross presentation, which promotes efficient vaccination, budding competent immunogens were designed. Assuming that Env expression could reduce Gag-specific immune responses, no vaccine variants coding for gp120 were developed.

The objective of this work was the detailed immunological characterization of the novel vaccine candidates in human *ex vivo* models. Initiation of cellular immune responses by direct presentation depends on efficient peptide presentation on antigen presenting cells (APC) which requires sufficient immunogen expression as well as APC maturation. Therefore, the novel vaccine candidates should be analyzed for induction of maturation and immunogen expression in monocyte derived dendritic cells (mdDC). Peptide presentation ought to be determined by restimulation of different HIV-Gag specific T cell clones.

Besides direct presentation, cross presentation represents an alternative way of generating CTL-based immune responses. Cross presentation requires availability of immunogens expressed by cells other than APC. Therefore, the vaccine candidates should be analyzed for induction of immunogen expression in epithelial HeLa cells and their capacity to be cross presented on APC which was analyzed by restimulation of Gag-specific CTL clones.

The obtained results will form the basis for further refinement of the immunogen candidates to yield an optimal next-generation T cell-vaccine directed against HIV-1.

C Material and methods

C.1 Material

C.1.1 Cells

C.1.1.1 Prokaryotic cells

The bacterial strains DH5α and DH10B were used for the amplification of plasmid DNA.

Tab. C.1 Bacterial strains

Strain	Genotype
DH5α	F^- *sup*E44 Δ*lac*U169 (φ80 *lac*ZΔM15) *hsd*R1 *rec*A1 *end*A1 *gyrA96 thi*-1 *rel*A1 [244]
DH10B	*ara*D139 Δ(*ara*,*leu*)7649 *gal*U *gal*K *rsp*L *nup*G (Gibco BRL)

C.1.1.2 Eukaryotic cell lines

All eukaryotic cell lines were maintained under conditions of 5% CO_2 and 37°C.

Tab. C.2 Eukaryotic cell lines

Strain	Description
DF-1	spontaneously immortalized chicken cell line derived from 10 day old East Lansing Line (ELL-0) eggs, ATCC: 12203 [245]
BHK-21	Hamster kidney fibroblast cell line, ATCC: CCL-10 [246]
HeLa	Human epithelial cell line derived from a cervix carcinoma, ATCC: CCL-2 [247]
LCL - HLA-A*2	EBV transformed lymphoblastoid cell line derived from an HLA-A*2 positive donor, Vaecgene Biotech GmbH [248]
LCL – HLA-B*07	EBV transformed lymphoblastoid cell line derived from an HLA-B*07 positive donor, Vaecgene Biotech GmbH [248]

C.1.2 Bacterial growth and cell culture media and buffers

Tab. C.3 Bacterial growth medium and buffers

Description	Ingredients
LB (lysogeny broth)	Yeast extract 0.5 % (w/v), tryptone 1 % (w/v), NaCl 1 % (w/v), pH 7.4
TB1 (Transformation buffer 1)	RbCl 100 mM, $MnCl_2$ 50 mM, KAc 30 mM, CaCl 10 mM, glycerine 15 %
TB2 (Transformation buffer 2)	MOPS 10 mM, RbCl 10 mM, CaCl2 75 mM, glycerol 15 %
RIPA buffer	Tris 50 mM, NaCl 150 mM, SDS 0.1 %, Na-desoxycholate 0.5 %, Triton X-100 1 %, pH 8,0 Complete Mini protease inhibitor (Roche, 1 tablet / 10 mL buffer)

Tab. C.4 Cell culture media and buffers

Description	Ingredients
DMEM	Dulbecco's modified Eagle's medium
DMEM-10	DMEM, FCS 10 % (Gibco), penicillin 100 U/mL, streptomycin 0.1 mg/mL
RPMI1640	Roswell Park Memorial Institute medium
RPMI1640-10	RPMI1640, FCS 10 % (Lonza), penicillin 100 U/mL, streptomycin 0.1 mg/mL
RPMI1640-10/50	RPMI1640, FCS 10 % (Lonza), Hepes 50mM, IL-2 10 U/mL, PHA-L 1 µg/mL, penicillin 100 U/mL, streptomycin 0.1 mg/mL
mdDC-medium	RPMI1640, FCS 10 % (Lonza), Pan Vitamins 1x, MEM 1x, L-glutamine 20 mM, pyruvate 1x, β-mercaptoethanol 0,05 mM, IL-4 500 U/mL, GM-CSF 500 U/mL, penicillin 40 U/mL, streptomycin 40 µg/mL
Storage medium	RPMI1640, FCS 50%, DMEM 10%, penicillin 100 U/mL, streptomycin 0.1 mg/mL
IMDM	Iscove's modified Dulbecco's medium
IMDM-8-10	IMDM, FCS 8% TCGF 10%, IL-15 10 ng/mL, penicillin 100 U/mL, streptomycin 0.1 mg/mL

Description	Ingredients
MACS buffer	PBS, BSA 0.5%, EDTA 2 mM
P2	PBS, FCS 2%
FACS buffer	PBS, FCS 1%, NaN_3 1 mg/mL
Perm / wash buffer	PBS, saponine 0.5%
Fixation buffer	PBS, saponine 1%, PFA 4%
TE buffer	Tris 10 mM, EDTA 1 mM, pH 7.5

C.1.3 HI-viral immunogens

The HI-viral immunogenes were derived from a C-clade 97CN54 or 97CN001 provirus clone collected in Sianking province (China) [243]. The gpn, gag and env genes were RNA and codon optimized by using the GeneOptimizer software package (Geneart AG, Regensburg; [249]).

Tab. C.5 Specificties of HIV-1 derived immunogens

Antigen		Specificities
gpn / gp120 (bidirectional) [88]	isolate	97CN54 ($Prot^-$)
	gp120	artificial signal peptide (MDRAKLLLLL LLLLLPQAQ), nucleotides: 5,673–7,109;
	gag	G2A mutation (Myr^-), nucleotides: 167–1,651;
	pol	5' part: D577N mutation ($Prot^-$), ∆rt nucleotides:1,444–2,406
		3' part: RT insertion, ∆int, nucleotides: 2,527–3,591;
		inserted in rt locus,
	nef	nucleotides 8,170–8,469 linked to 3′ end of nucleotides 8,470–8,787
gpn (∆FS, Myr^-)	isolate	97CN001
	gag	G2A mutation (Myr^-), nucleotides: 167–1,651;
	pol	5' part: ∆rt nucleotides:1,444–2,406
		3' part: RT insertion, ∆int, nucleotides: 2,527–3,591;
	nef	inserted in rt locus,
		nucleotides 8,170–8,469 linked to 3′ end of nucleotides 8,470–8,787
gpn (FS, Myr^-)	isolate	97CN001
	gag	G2A mutation (Myr^-), nucleotides: 167–1,651;

Antigen		Specificities
	FS	inserted at positions 1293-1345 in the gpn construct
	pol	5' part: ∆rt nucleotides:1,444–2,406
		3' part: RT insertion, ∆int, nucleotides: 2,527–3,591;
	nef	inserted in rt locus,
		nucleotides 8,170–8,469 linked to 3′ end of nucleotides 8,470–8,787
gpn (FS, Myr^+)	isolate	97CN001
	gag	nucleotides: 167–1,651;
	FS	inserted at positions 1,293–1,345 in the gpn construct
	pol	5' part: ∆rt nucleotides:1,444–2,406
		3' part: RT insertion, ∆int, nucleotides: 2,527–3,591;
	nef	inserted in rt locus,
		nucleotides 8,170–8,469 linked to 3′ end of nucleotides 8,470–8,787
Gag (Myr^-) [249]	isolate	97CN001
		G2A mutation (Myr^-), nucleotides: 167–1,651
Gag (Myr^+) [249]	isolate	97CN001
		nucleotides: 167–1,651

C.1.4 Viruses

C.1.4.1 General

NYVAC (New York Vaccinia virus) is a highly attenuated virus strain. It was derived from the Copenhagen vaccinia strain by the deletion of 18 ORF that influence virulence, host range and pathogenesis of the virus [78].

C.1.4.2 NYVAC with HIV-1 derived transgenes

The HIV-1 transgenes (see C.1.3) were inserted by recombination at the J2R locus under the control of a vaccinia early / late promoter.

Tab. C.6 Properties of the NYVAC based vaccine candiates

Description	HIV frameshift	Myristoylation site	Transgene specifications
NYVAC-C	-	-	Expression of HIV-1 gp120 and GPN polyprotein under the control of a bidirectional promoter, no VLP budding
NYVAC-gpn (ΔFS, Myr^-)	-	-	Expression of the GPN polyprotein, no VLP budding
NYVAC- gpn (FS, Myr^-)	+	-	Expression ratio of 95 % Gag and 5% GPN (estimated) [250], no VLP budding
NYVAC- gpn (FS, Myr^+)	+	+	Expression ratio of 95 % Gag and 5% GPN (estimated) [250], VLP budding
NYVAC-gag (Myr^-)		-	Expression of HIV-1 Gag, no VLP budding
NYVAC-gag (Myr^+)		+	Expression of HIV-1 Gag, VLP budding
NYVAC-gp120			Expression of HIV-1 gp120,
NYVAC-wt			no transgene

C.1.5 DNA

C.1.5.1 Oligonucleotides

Tab C.7 Oligonucleotides for sequence analysis

Description	Sequence (5'→3')	Transgene
97CN001-SynGag-SQP_402 fw	CCTGCAGGGCCAGATGGTGC	gpn/gag
97CN001 580R	GTCCTTCAGGATCTGCATAG	gpn/gag
Syn-GPN_681 fw	GCCCAGGGGCAGCGACATCG	gpn/gag
GPN 1280F	CAGATGAAGGACTGCACCGA	gpn/gag
GPN 1780F	CGTGCTGGAGGACCTGAACC	gpn
GPN 2290F	GAAGAAGAGCTGACCGTGC	gpn
GPN 2790F	CCGAGTTCTACAAGGACTGC	gpn
GPN 3280F	CAAGCTGAACTGGGCCAGCC	gpn
CN54 3701F	AGTGGGAGTTCGTGAACACC	gpn
97CN001-SynGag-SQP_901fw	CTGCGGGCCGAGCAGGCCAC	gpn
C-synenv 1208(120b)	GGTGGTGGCGCCCTTCCACAC	gp120
CN54_Env_620F	TGCAACACCAGCGCCATCAC	gp120
Env 320R	CGCAGGGCTTCAGGCTCTGG	gp120
97CN001_290F	GAGATCGACGTGCGGGACAC	gp120
CN54_Env_1120F	CAGCGGCGGCGACCTGGAGG	gp120

C.1.5.2 Vectors

Tab. C.8: Commercial DNA-Vectors

Description	Specification (Supplier)
pcDNA3.1(+)	Eukaryotic expression plasmid (Invitrogen, Karlsruhe)
pcDNA3.1(-)	Eukaryotic expression plasmid (Invitrogen, Karlsruhe)

Tab. C.9: Properties of plasmids coding for HIV-1 derived antigens

Description	HIV frameshift	Myristoylation site	Transgene specifications
pcDNA-3.1(+)_gpn (ΔFS, Myr^-)	-	-	Expression of the GPN polyprotein, no VLP budding
pcDNA-3.1(+)_gpn (FS, Myr^-)	+	-	Expression ratio of 95 % Gag and 5% GPN (estimated) [250], no VLP budding
pcDNA-3.1(+)_gpn (FS, Myr^+)	+	+	Expression ratio of 95 % Gag and 5% GPN (estimated) [250], VLP budding
pcDNA-3.1(-)_gag (Myr^-)		-	Expression of HIV-1 Gag, no VLP budding
pcDNA-3.1(-)_gag (Myr^+)		+	Expression of HIV-1 Gag, VLP budding
pcDNA-3.1(+)_gp120			Expression of HIV-1 gp120,
NYVAC-gfp			Expression of humanized green fluorescent protein

C.1.6 Antibodies

C.1.6.1 Western blot antibodies

Tab. C.10: Primary and secondary Western blot antibodies

Description	Supplier / Company	Specifications
CB-4/1	Charité Berlin, Prof. Dr. W. Höhne	Monoclonal mouse Ab raised against HIV clade C capsid protein (p24) Dilution 1:1000
Anti-mouse IgG HRP-conjugated	Dako, Hamburg	HRP conjugated goat Ab raised against mouse IgG Dilution: 1:2000

C.1.6.2 FACS antibodies

Tab. C.11: FACS antibodies for ICS and surface protein staining

Description	Supplier / Company	Specifications
KC-57 RD1	Beckman Coulter	Monoclonal mouse Ab raised against HIV clade C capsid protein (p24) Volume / test: 2µl
Anti-human CD80 FITC-conjugated	BD Pharmingen	Monoclonal mouse Ab raised against human CD80 Volume / test: 2µl
Anti-human CD86 V450-conjugated	BD Pharmingen	Monoclonal mouse Ab raised against human CD86 Volume / test: 3µl
Anti-human HLA-DR APC-conjugated	BioLegend	Monoclonal mouse Ab raised against human HLA-DR Volume / test: 4µl
Anti-human CD8 APC-Cy7-conjugated	BD Pharmingen	Monoclonal mouse Ab raised against human CD8 Volume / test: 3µl
Anti-human IFN-γ FITC-conjugated	BD Pharmingen	Monoclonal mouse Ab raised against human IFN-γ Volume / test: 2µl

C.1.7 Cytokines and growth factors

Tab. C.12: Cytokines and growth factors

Description	Supplier / Company	Specifications
Human IL-2	Miltenyi Biotec GmbH	50 U/mL
Human IL-4	Cellgenix Technology Transfer Gmbh	500 U/mL
Human IL-15	Cellgenix Technology Transfer Gmbh	10 ng/mL
TCGF	Helvetica Healthcare	Final concentration: 10% in RPMI
GM-CSF (Leukine™)	Genzyme	500 U/mL

C.1.8 Cell culture agents

Tab. C.13: Cell culture agents

Description	Supplier / Company	Specifications
PHA-L	Sigma Aldrich	1 µg/mL
BFA	Biochrom AG	1 µg/mL
LPS	Sigma Aldrich	100 ng/mL

C.1.9 Peptides

Tab. C.14: Synthetic peptides for CTL stimulation

Description	Supplier / Company	Specifications
GPGHKARVL (GL9)	Biosynthan GmbH	Origin: HIV-1 Gag p24 Residues: 223-231 Restriction: HLA-B*07 Concentration: 3 µg/mL
SLFNTVATP (SP9)	Biosynthan GmbH	Origin: HIV-1 Gag p17 Residues: 77-85 Restriction: HLA-A*2 Concentration: 3 µg/mL
SLYNTVATL (SL9)	Biosynthan GmbH	Origin: HIV-1 Gag p17 Residues: 77-85 Restriction: HLA-A*2 Concentration: 3 µg/mL

C.1.10 Commercial kits

Tab. C.15: Commercial kits

Description	Supplier
Endotoxin free DNA preparation Maxi kit	Qiagen Hilden
Amobocyte Lysate QCL-1000 kit	Lonza Group LTD

C.1.11 Chemicals and plastics

If not further specified, all chemicals were purchased Sigma and Merck, cell culture flasks as well as general plastic material from BD Falcon and cell cultural supplies by PAN Biotech, GibcoBRL and Lonza Group LTD.

C.2 Microbiological techniques

C.2.1 Growth and selection of bacteria

Bacterial cultivation was performed overnight in LB medium at 37°C. For the selection of transformed bacteria, LB medium with 100 µg/mL ampicillin was used.

C.2.2 Generation of chemically competent bacteria

Chemically competent bacteria were obtained by the use of the RbCl method [244]. All steps were performed at 4°C, centrifugation steps were performed at 2000 x g for 15 min.
50 mL of an *E.coli* overnight culture were inoculated in 450 mL LB medium and incubated at 37 °C until reaching an optical density of OD_{600} = 0,4. After centrifugation, the bacteria were resuspended in 16 mL chilled TB1 and incubated on ice for 15 min. Afterwards the cells were centrifuged and the pellet was resuspended in a final volume of 3 mL cooled TB2, divided in 100 µL aliquots and stored at -80°C.

C.2.3 Transformation of chemically competent bacteria

For the transformation 100 µL of chemically competent bacteria (C.2.2) were thawed on ice, mixed with 1 µg plasmid DNA and incubated on ice for 10 min. For transformation, the bacterial cell wall was permeabilized by heat induction at 42°C for 1 min, followed by incubation on ice for 1 min. After addition of 500 µL LB medium, the cells were incubated at 37 °C for 1 h with shaking at 300 rpm and subsequently plated on LB agar selection plates containing appropriate antibiotics.

C.3 Molecular biology techniques

C.3.1 DNA purification

To avoid contaminations with immune modulatory bacterial components such as LPS (lipopolysaccharide) in cross presentation studies, endotoxin-free plasmid DNA was generated using an Endotoxin-free DNA preparation Maxi kit (C.1.10).
The eluted DNA was resuspended in TE-buffer and the concentration was measured by photometric analysis (Nanodrop-1000 spectrophometer, Peqlab). The LPS content was determined with the Amobocyte Lysate QCL-1000 kit (C.1.10).

C.3.2 PCR analysis

The inserted transgenes (C.3.1) were verified by polymerase chain reaction (PCR) [251] analysis. The DNA was screened using adequate primer pairs (C.1.5) and the 2x PCR Master Mix (Promega, Madison, USA). The DNA was amplified in 25 cycles (denaturation 94 °C, 30 s; Annealing 58 °C, 30 s; extension 72 °C, 60 s; final elongation 72 °C, 15 min).
To determine PCR efficiency the generated DNA fragments were analyzed for correct size by agarose gel-electrophoresis (1% agarose in TBE buffer; Tris 89mM, boric acid 89mM, EDTA 2mM, ethidium bromide 0.01%).

C.4 Protein biochemistry techniques

C.4.1 Sample processing for protein immunostaining

For protein analysis by immunostaining, secreted proteins have to be concentrated (C.4.1.1), intracellular proteins have to be released from the cells (C.4.1.2). All sonication steps were done with a Sonifier 450 (Branson) at 50 % exposure time and 80 % max. output power. The protein concentration was determined prior to use in immunostaining assays by spectrophotometry (C.4.1.3) [252].

C.4.1.1 Concentration of secreted proteins

For concentration of secreted proteins, the supernatant of infected cells was collected and Na-desoxycholate was added to a final concentration of 0,2%. After incubation for 5 min at 4°C, the sample was sonicated for 1 min and mixed with TCA to a final concentration of 5,5% TCA. The aggregated proteins were pelleted (8.000 x g, 10 min, 4°C) and subsequently resuspended in a desired volume of dH_2O.

C.4.1.2 Cell lysis

To analyze intracellular proteins by immunostaining, the cells were washed with PBS, harvested and pelleted (2.000 x g, 10 min, 4°C). The pellet was resuspended in 100 µL RIPA buffer (C.1.2), incubated for 1 h at 4°C and sonicated for 1 min for complete cell lysis. Subsequently, the cellular debris and insoluble components were pelleted by centrifugation (2.000 x g, 10 min, 4°C) and the protein concentration of the supernatant was determined (4.1.3).

C.4.1.3 Determination of protein concentrations

The concentration of total protein amount was determined using the protein assay according to Bradford by measuring the 595 nm absorption of proteins bound to chromogenic substrates [252]. The proteins were stained with the BioRad protein assay (BioRad, Munich) according to the manufacturer's instructions. For protein quantification, a calibration line based on the linear regression model was determined with BSA solutions of distinct concentrations.

C.4.2 Protein immunostaining

C.4.2.1 SDS-PAGE and Western blot analysis

Electrophoretic protein separation was performed as described by Laemmli [253]. Protein samples of cell lysates (4.1.2) and supernatants (4.1.1) were mixed with 2x sample buffer (Tris/HCl 120mM pH 6,8, SDS 2 % (w/v), β-mercaptoethanol 10 %, EDTA 1 mM, glycerol 10 % (w/v), bromophenol blue 0,01 % (w/v)) and heated for 5 min at 95°C.

The separated proteins were then transferred onto nitrocellulose membranes by Western blotting using a semi-dry blotting device (Serva Electrophoresis GmbH) according to manufacturer's instructions. Subsequently the nitrocellulose membrane was incubated for 1 h in MTBS buffer (NaCl 150 mM, Tris/HCl 50 mM, skim milk powder 5 % (w/v), pH 7.4) to saturate unspecific antibody binding areas on the membrane. For protein immunostaining, the primary and secondary antibodies (C.1.5) were diluted appropriately in MTBS buffer, incubated for 1 h and subsequently washed three times with TTBS buffer (NaCl 150 mM, Tris/HCl 50 mM, Tween-20 0,3 %, pH 7.4). Protein bands were visualized by enhanced chemiluminescence using SuperSignal West Pico Chemiluminescent Substrate (Thermo Solutions) according to the supplier's instructions and detected with a ChemiluxPro device (Intas Science Imaging Instruments GmbH, Göttingen).

C.4.2.2 Flow cytometry analysis (FACS)

The specific detection of proteins by flow cytometry was performed in 96-well U-formed reaction plates, centrifugation steps were done at 300 x g for 5 min at 4 °C. $1x10^4$ cells were analyzed in a FACS Canto II device (BD, FACS Diva v6.0 software).

C.4.2.2.1 Detection of surface proteins in FACS analysis

For immunostaining of proteins expressed on the cell surface, cells were pelleted and washed twice in 200 µl chilled FACS buffer to interrupt the cell metabolism. The incubation of the specific antibodies (C.1.5.3) was done for 25 min at 4°C under light exclusion in 50 µL FACS buffer. After

staining, unbound antibodies were removed by two washing steps with 200 µl FACS buffer. For analysis or storage, the cells were resuspended in 100 µl chilled FACS buffer.

C.4.2.2.2 Detection of intracellular proteins / Intracellular cytokine staining (ICS)

For intracellular detection of regularly secreted proteins such as cytokines by FACS analysis, cells were cultivated with the intracellular transport blocking agent Brefeldin A (BFA, 0.1 µg/mL, C.1.8). Cells were pelleted by centrifugation and washed twice in 200 µl chilled FACS buffer to interrupt the cell metabolism [254]. After treatment with buffer cells were washed twice with 100 µl Perm / Wash buffer (C.1.2). The appropriate antibodies (C.1.5.3) were diluted in 50 µL Perm / Wash buffer and incubated for 25 min at 4°C under light exclusion. To remove unbound antibodies cells were washed four times with 200 µL Perm / Wash buffer. For analysis or storage, the cells were resuspended in 100 µl chilled FACS buffer.

C.5 Cell culture techniques

C.5.1 General infection procedures

C.5.1.1 Generation and maintenance of mdDC

Monocyte derived cells (mdDC) were generated by a modified protocol described by Thurner [255]. For the generation of approximately $1x10^7$ mdDC, PBMC were isolated from a 50 mL blood sample using Leucosep tubes (Greiner Bio-one) according to the supplier's manual. The buffy coat was washed twice with P2 buffer (C.1.2), the cell count was determined in a Vi-Cell device (Beckman Coulter). Monocytes were isolated with CD14 microbeads in a MACS assay (Miltenyi Biotec GmbH) according to the manufacturer's instructions. For cell differentiation, the obtained cells were counted, set to a cell density of $1x10^6$ / mL and incubated for 5 - 6 d in mdDC medium (C.1.2) supplemented with IL-4 and GM-CSF (C.1.6).

C.5.1.2 Proliferation assay for CTL cell clones

Cytotoxic T lymphocytes (CTL) were proliferated with HLA matching LCL feeder cells (Vaecgene Biotech GmbH). After incubation with the corresponding peptide (C.1.8) for 2-3 h at 37°C in RPMI1640-10 medium (C.1.2; 1 mL/$1x10^7$ cells), the LCL cells were irradiated with 60 gy in a gamma ray device and subsequently diluted in IMDM-8-10 medium (C.1.2; 10 mL/$1x10^7$ cells) with IL-15 (C.1.6). The feeder cells were cocultivated with $1x10^6$ freshly thawed CTLs. The solution was transferred into 96-well U-formed reaction plates (100 µL/well) and incubated at 37 °C and 5 % CO_2. Medium was refreshed in a three-day repetition by exchanging 50 µL

medium. After a 14-day period, the CTL were frozen using an Ice Cube 14S cell freezer device (SY-LAB) in storage medium (see. C.1.2).

C.5.1.3 Transfection of human cell lines

Human cell lines were transfected with Lipofectamine 2000 (Invitrogen, Life Technologies) according to the manufacturer's instructions.

C.5.1.4 Infection of human cell lines with NYVAC

For the infection of human cell lines with NYVAC, cells were seeded in DMEM-10 medium (C.1.2) either concentrated at $5x10^5$ cells/well in a 6 well reaction plate or at $5x10^4$ in a 24-well flat bottom plate. Prior to infection, DMEM-10 was replaced by DMEM medium (C.1.2), the virus was homogenized by sonication for 1 min in a Sonifier 450 device (C.4.1) and subsequently mixed on a vortex device. 1 h after infection with the desired multiplicity of infection (MOI), unbound and loosely attached viruses were removed by stringent washing with 5 x 1 mL PBS. For further cultivation DMEM-10 medium was used.

C.5.1.5 Infection of mdDC with NYVAC

For the infection of mdDC with NYVAC, $5x10^4$ cells/well of 5 d old mdDC were seeded into a 96-well reaction plate in 100 µl DC-medium (C.1.2). Prior to infection, the virus was homogenized by sonication for 1 min in a Sonifier 450 device (C.4.1) and subsequently mixed on a vortex device. The cells were infected with the desired MOI in 100 µl DC-medium and incubated for 1 h. Unbound and loosely attached viruses were removed by stringent washing with 5 x 1 mL PBS, after each washing step cells were pelleted by centrifugation at 500 x g for 5 min at 4 °C. For further cultivation, DC-medium was used.

C.5.2 Generation of NYVAC virus stocks

C.5.2.1 Infection of BHK cells

NYVAC stocks (C.1.4.3) with high virus titers were generated in baby hamster kidney cells (BHK, C.1.1). $2x10^7$ cells were cultivated in a 175 cm^2 cell culture flask for 6 h and subsequently infected with NYVAC (MOI 0.5 – 3) for 2 h in 15 mL DMEM medium (C.1.2). After removal of the medium, the cells were incubated for 48 h in DMEM-10 (C.1.2).

C.5.2.2 Virus purification

To release intracellular viruses, the infected BHK cells (see C.5.2.1) were scraped from the cell culture flask and pelleted at 2000 x g for 15 min at 4°C. The cells were resuspended in 1 mL Tris-HCl (Tris 10 mM, pH 9) and immediately disrupted by three freezing/thawing cycles. After sonication for 2 x 2 min with a Sonifier 450 device (C.4.1) cell debris were pelleted at 2000 x g for 15 min at 4°C. Afterwards, the supernatant was transferred to a new vial, sonicated for 1 x 1 min and pelleted again. The supernatants were then combined and diluted in 16 mL Tris-HCl, the viruses were concentrated by ultracentrifugation (Optima L-90K, SW32 Ti rotor, Beckman Coulter) in a sucrose density gradient (sucrose 45 % w/v, Tris 10 mM, pH 9) twice. For gradient generation the sucrose solution was covered with the virus suspension in centrifugation tubes (25 mm x 89 mm, Beckman Coulter) and centrifuged for 1 h at 20,000 x g at 4 °C. The virus pellet was resolved in 500 µL Tris-HCL, aliquoted and stored at -80 °C.

C.5.2.3 NYVAC titration

After freezing at -80°C the generated NYVAC stocks (C.1.4.3) were titrated in a plaque forming assay. DF-1 cells (C.1.1) were cultivated with a density of $7x10^5$ cells/well in a 6-well plate for 6 h in DMEM-10 medium (C.1.2). For infection, the medium was removed and serial dilutions of the virus suspension (C.5.2.2) in 400 µL DMEM medium (C.1.2) were incubated on the cells for 1 h. The cells were maintained for 2 d with 1 mL DMEM-10. For virus quantification, the viral plaques were visualized by staining of the confluent cells for 10 min with crystal violet solution (formol 25 % (v/v), ethanol 8,5 % (v/v), crystal violet 1.5 g/L). The virus titer was obtained by determining the number of plaques considering the dilution factor.

To analyse NYVAC replication in HeLa cells, $1x10^5$ cells were cultivated in 24-well culture plates. For infection, DMEM-10 was exchanged by 100 µL DMEM medium. To analyse NYVAC replication in mdDC, $1x10^5$ cells diluted in by 100 µL RPMI1640 medium were disseminated in 24-well culture plates. The cells were infected with different vaccine candidates at MOI 1 and incubated for 1 h. Viruses loosely attached to the cell membrane and present in the supernatant were removed six washing cycles with PBS. To analyze time dependency, replication was determined in 4 h time periods (up to 24 h). After cultivation, cells and supernatant were sonicated for 2 x 2 min with a Sonifier 450 device (C.4.1). For infection the solution was diluted in an appropriate amount of DMEM. Further titration was performed as described above.

C.5.3 Transgene expression after NYVAC infection and transfection

The expression rate of different HIV transgenes (C.1.4.3) was determined by p24 staining. HeLa cells and mdDC were infected with NYVAC or transfected as described above (C.5.1.3, C.5.1.4 and C.5.1.5). At indicated time points post infection (h p.i.), the cells were permeabilized, stained (C.4.3.2.3) with an anti-p24 antibody (C.1.5.3), and analyzed by FACS analysis (C.4.2.3).

C.5.4 Maturation of mdDC after NYVAC infection

The mdDC maturation elicited by infection with NYVAC was determined by analysis of upregulation of the maturation markers CD80, CD86 and HLA-DR. mdDC were infected and stained as described above (C.5.1.5, C.4.2.3.1 and C.1.5.3). mdDCs, pulsed with LPS (C.1.7) served as positive control.

C.5.5 Direct presentation of antigenic peptides on mdDC

To activate HIV-1 specific CTL by direct presentation, cells were infected with NYVAC (C.1.4.3) as described (C.5.1.5). First, the optimal direct presentation time point was evaluated by starting the CTL coculture at varying times. For the following experiments, the CTL were cocultivated 4 h after mdDC infection. The CTL were thawed and adjusted to a concentration of $5x10^5$ cells/mL in RPMI1640-10 (C.1.2) 4 h prior to the coculture start point. Immediately before coculturing, BFA was added to the CTL to interrupt the secretion of IFN-γ (C.1.7). After a 12 h coculturing period, the cells were harvested and stained in an ICS assay (see C.4.2.2.2) with an anti-IFNγ antibody (C.1.5.3).

C.5.6 Cross presentation of antigenic peptides on mdDC

For determination of the cross presentation potential of the different HIV-1 Gag derived transgenes (C.1.3), $5x10^4$ HeLa cells were either transfected with plasmids coding for various HIV derived antigens (C.1.5.2) or infected with recombinant NYVACs (C.1.4.3). 100 µL mdDC in a concentration of $5x10^5$ cells/mL in RPMI1640-10 (C.1.2) were cocultivated with the HeLa cells 4 h post infection. HIV-1 specific CTLs were cocultivated as described before (C.5.6).

To differentiate between cross presentation mediated by extracellular proteins or infected cells, HeLa cells and supernatant were separated 4 h post NYVAC infection by centrifugation for 5 min at 500 x g. The coculture with mdDC was performed either with the supernatant or cells resolved in 100 µL RPMI1640-10 (C.1.2). After a 12 h coculturing period, the cells were harvested and stained in an ICS assay (C.4.2.3.2) with an anti-IFNγ antibody (C.1.5.3).

D Results

D.1 Design of antigens with optimized immunogenicity

The objective of the EuroVacc foundation was the development of a HIV-1 vaccine that induces effective Gag-specific CTL responses. In the phase-I clinical trials EuroVacc01 and EuroVacc02, the vaccine candidate NYVAC-C induced only modest Gag, Pol and Nef specific CTL responses (see B.2.2.3).

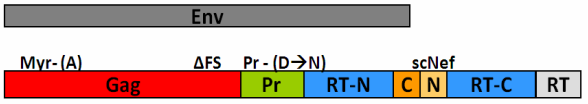
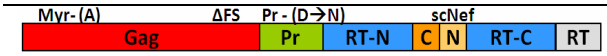
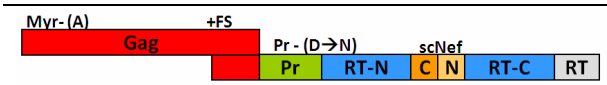
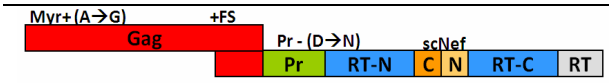
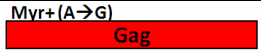
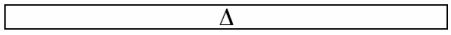
To enhance immunogenicity, a new generation of antigens based on the immunogen 97CN54 GPN was developed [256]. These new generation antigens were designed to (i) induce increased Gag expression, (ii) be accessible for cross presentation and (iii) circumvent replacement of Gag epitopes by immunodominant Env, Pol or Nef peptides (see table D.1). To realize efficient VLP formation, seven amino acids were substituted in all new generation antigens, resulting in an isolate switch from 97CN54 to 97CN001 derived from the same patient. The gpn, gag and env genes were RNA and codon optimized by using the GeneOptimizer software package (Geneart AG, Regensburg; [249]) comprising codon usage adaption and deletion of deleterious sequence elements. To realize the required antigen characteristics, the following modifications were made:

1. A vaccine variant without gp120 is favoured to avoid immunodominant effects of HIV-Env. Secondly, elimination of gp120 circumvents Env-mediated apoptosis and therefore could result in enhanced antigen production in transgenic cells [257] [gpn (ΔFS, Myr$^-$), see Tab. D.1].
2. The reinsertion of a ribosomal frame shift site by reconstitution of the initial codon usage at the amino acid positions 1293 to 1345 induces a -1 reading frame switch in 5% of all translation events [250]. When ribosomal re-positioning occurs, the mRNA is translated across the frame shift region resulting in GPN expression. Hence, the frame shift region induces translation of Gag and GPN in a ratio of 95:5 leading to an elevated intracellular Gag concentration [gpn (FS, Myr$^-$) and gpn (FS, Myr$^+$), see Tab. D.1].
3. For enhancing immunogenicity or when applying vector systems not affecting APC, efficient induction of T cell responses demands cross presentation. The amount of extracellular antigen can be enhanced by utilizing the natively occurring VLP formation of HIV-1 Gag molecules (see B.2.2.2). To enable VLP budding, myristoylation of Gag is required. To restore the myristoylation acceptor site, the G2A mutation was reverted [gpn (FS, Myr$^+$) and gag (Myr$^+$), see Tab. D.1]. The obtained immunogens were shown to induce VLP formation efficiently [256].
4. To enhance Gag-specific T cell responses further, the polnef gene sequence was eliminated in some immunogens. The resulting antigens eludes the replacement of

Gag-derived peptides by immunodominant PolNef epitopes, but at the expense of a minimized number of epitopes in total [gag (Myr^-) and gag (Myr^+), see Tab. D.1].

For the generation of vaccine candidates based on the new generation antigens, NYVAC vectors were generated. In contrast to the NYVAC-C vaccine, where the gp120 and GPN transgenes are controlled a bidirectional E/L promoter, a monodirectional E/L promoter is used in the novel vaccine variants. The immunogens and vaccine candidates were developed and kindly provided by Dr. Simon Bredl, Dr. Josef Köstler, Katharina Böckl and Dr. Kathrin Kindsmüller (Molecular Microbiology and Gene Therapy Unit, Institute of Medical Microbiology and Hygiene, University of Regensburg, Head of Department Prof. Dr. Ralf Wagner). The objective of this work was the detailed immunological characterization of the novel vaccine candidates in human *ex vivo* systems. The induced Gag expression was analyzed in both, epithelial and dendritic cells (see D.2). Furthermore, the immunogen's influence on DC maturation was analyzed (see D.3). To determine the immunogenicity of the resulting new generation vaccine candidates, their ability to be presented by direct and cross presentation was examined (see D.4, D.5, D.6 and D.7). Additionally, the influence of the delivery system on antigen presentation was determined (see D.5).

Tab. D.1 Schematic illustration of immunogen composition, modifications and nomenclature

Antigen illustration	Expressed antigens	VLP formation	Nomenclature
	gp120 GPN (equimolar)	Ø	-C
	GPN (100 %)	Ø	-gpn (ΔFS, Myr^-)
	Gag (95 %) GPN (5 %)	Ø	-gpn (FS, Myr^-)
	Gag (95 %) GPN (5 %)	+	-gpn (FS, Myr^+)
	Gag	Ø	-gag (Myr^-)
	Gag	+	-gag (Myr^+)
	gp120		-gp120
	Ø		wt

D.2 Antigen expression in NYVAC infected cells

To enhance their immunogenicity, the new generation antigens were designed for maximal expression in infected cells (see D.1). Long lasting immunity and high-level antigen expression were believed to be favourable for efficient direct and cross presentation of antigen-derived peptides. To determine antigen expression of the different the vaccine candidates, HIV-1 Gag p24 was quantified in HeLa cells by Western blot and FACS analysis. To determine optimal conditions for the cross presentation assay, the influences of virus concentration and incubation time on the intracellular Gag-p24 steady-state level were investigated.

D.2.1 HIV-1 Gag based antigens are expressed in large amounts in NYVAC infected HeLa cells

For the development of a vaccine eliciting efficient T cell responses, cross presentation is a required feature [141,142]. Particularly, when applying vector systems not affecting APC, presentation of extracellularly derived antigens is needed for CTL activation. Epithelial cells represent one of the cell types that first encounter antigen in a vaccination. Therefore, vaccine-induced immunogen expression in epithelial cells represents a source of antigen accessible for presentation on DC. For cross presentation assays, the human epithelial tumor cell line HeLa was utilized as a model system.
To determine the influences of (i) possible gp120 cytotoxicity, (ii) reduced GPN expression, (iii) VLP formation and (iv) the deletion of PolNef epitopes, infected HeLa cells were analyzed for immunogen expression in Western blot and FACS assays.

Due to their potential cytotoxicity the developed immunogens are prone to nonsense mutations. Therefore, full-length expression was monitored in a Western-blot analysis. HeLa cells were infected at MOI = 5 and incubated for 48 h. Immunogen expression was detected intracellularly. Additionally, VLP formation was observed by detection of p55 in the culture supernatants. Expression of the artificial 160 kDa GPN polyprotein could be detected in NYVAC-C and NYVAC-gpn (ΔFS, Myr^-) infected cells (see Fig. D.1). Obviously, NYVAC-C induced weak immunogen expression whereas in NYVAC-gpn (ΔFS, Myr^-) infected cells, an increased steady-state level of GPN was detected. A ribosomal frame shift led to GPN amounts below the limit of detection. Immunogen size-reduction by Pol-Nef deletion did not enhance p55 expression in NYVAC-infected cells. NYVAC-gpn (FS, Myr^-) and NYVAC-gpn (FS, Myr^+) elicited intracellular steady-state levels comparable to those induced by NYVAC-gag (Myr^-) and NYVAC-gag (Myr^+). Budding competence reduced the intracellular immunogen amount. Extracellular p55 was detected in the supernatant of NYVAC-gpn (FS, Myr^+) and NYVAC-gag (Myr^+) infected HeLa cells. Surprisingly, the budding incompetent Gag (Myr^-) immunogen was detected extracellularly 48 h post

infection (hpi). The presence of budding incompetent Gag might be caused by the high antigen expression levels. Extensive expression in combination with long incubation period -required for Western-blot detection – is likely to induce necrosis in the target cells.

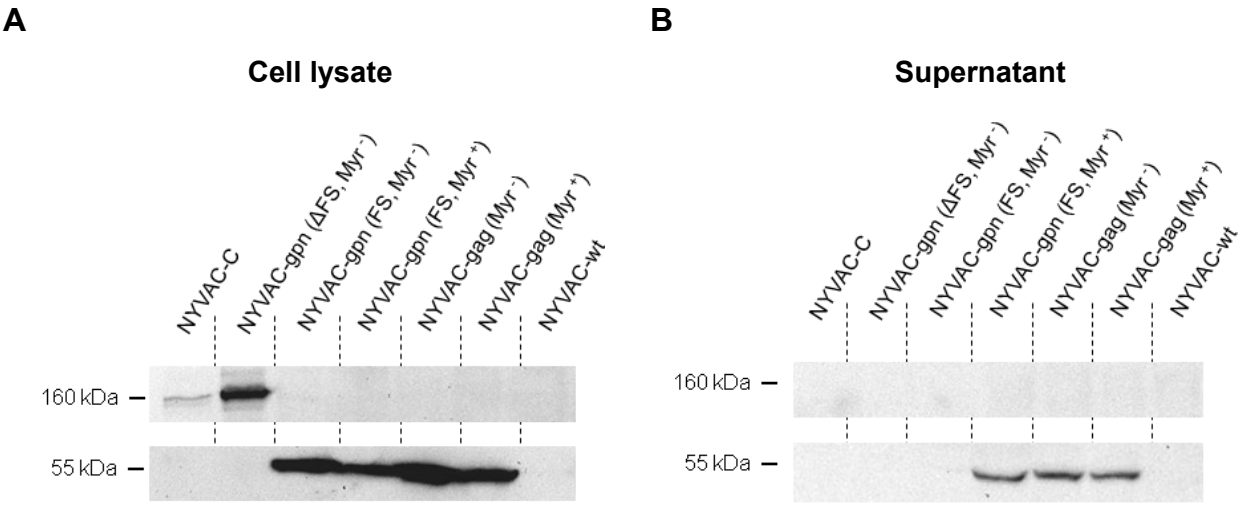


Fig D.1 The vaccine candidates elicit antigen expression in infected HeLa cells

HeLa cells were infected with different vaccine candidates at MOI 5 and harvested 48 hpi. For immunostaining, total cell extracts and concentrated supernatants were prepared. 60 µg of the total protein were applied in an SDS-PAGE. The proteins were transferred to a nitrocellulose membrane and detected with a primary anti-Gag p55 monoclonal antibody and a secondary goat anti-mouse HRP conjugated antibody. *A*, Detection of intracellular GPN and Gag; *B*, Detection of GPN and Gag in the supernatant

To further analyze the immunogen expression characteristics, HeLa cells were infected with different vaccine candidates by two different concentrations. After incubation for varying time periods the cells were stained with an anti-p24 antibody. The target cells were analyzed to express the corresponding immunogens by FACS analysis.

To analyze the infectivity of the different vaccine candidates, the number of p24 positive cells was quantified (see Fig. D2). Additionally, the immunogens' expression levels were analyzed by the mean fluorescence intensity (MFI, see Fig. D3 and D4).

In FACS analysis up to 90% of the infected cells were detected to be p24 positive (see Fig. D.2). Interestingly, Gag expression could be detected remarkably early. At both low (MOI = 0.5) and high (MOI = 5) MOI infections, the antigen was detected intracellularly 4 hpi. The number of p24 expressing cells did not vary significantly among the vaccine candidates.

At low MOI infections, after detecting a first expression maximum 4 hpi the intracellular Gag amount slightly decreased 6 hpi. When completing the first NYVAC replication cycle within a period of 8 - 12 h, p24 expression increased strongly. Although NYVAC replicates poorly in HeLa cells, virus-replication resulted in generation of a virus concentration outreaching those primarily employed. Due to reinfection with the newly generated NYVACs, an elevated number of p24 positive cells was detected after one replication cycle 12 hpi.

Due to the superior virus number at high MOI infections, an unsynchronized infection occurred. Therefore, the transgene expression course was not influenced by NYVAC replication cycle in high MOI infections.

With increasing virus concentrations, variations in the number of p24 positive cells emerged. Although NYVAC infectivity is assumed not being influenced by the transgene itself, for some vaccine candidates such as NYVAC-gag (Myr^-), an increased number of Gag positive cells was detected. Due to weak antigen expression induced e.g. by NYVAC-C, in some cells a p24 amount below limit of detection can be suggested. Thus, cells expressing a small antigen amount were not detected to contain HIV-Gag. Taken together, from an elevated number of p24 positive cells an increased Gag expression can be concluded.
In summary, it was shown that the antigens optimized for Gag expression fulfilled the desired requirements. (i) Deletion of gp120, (ii) insertion of an HIV frame shift, and (iii) elimination of PolNef resulted in increased number of p24 positive cells. For further discussion, see E.1.

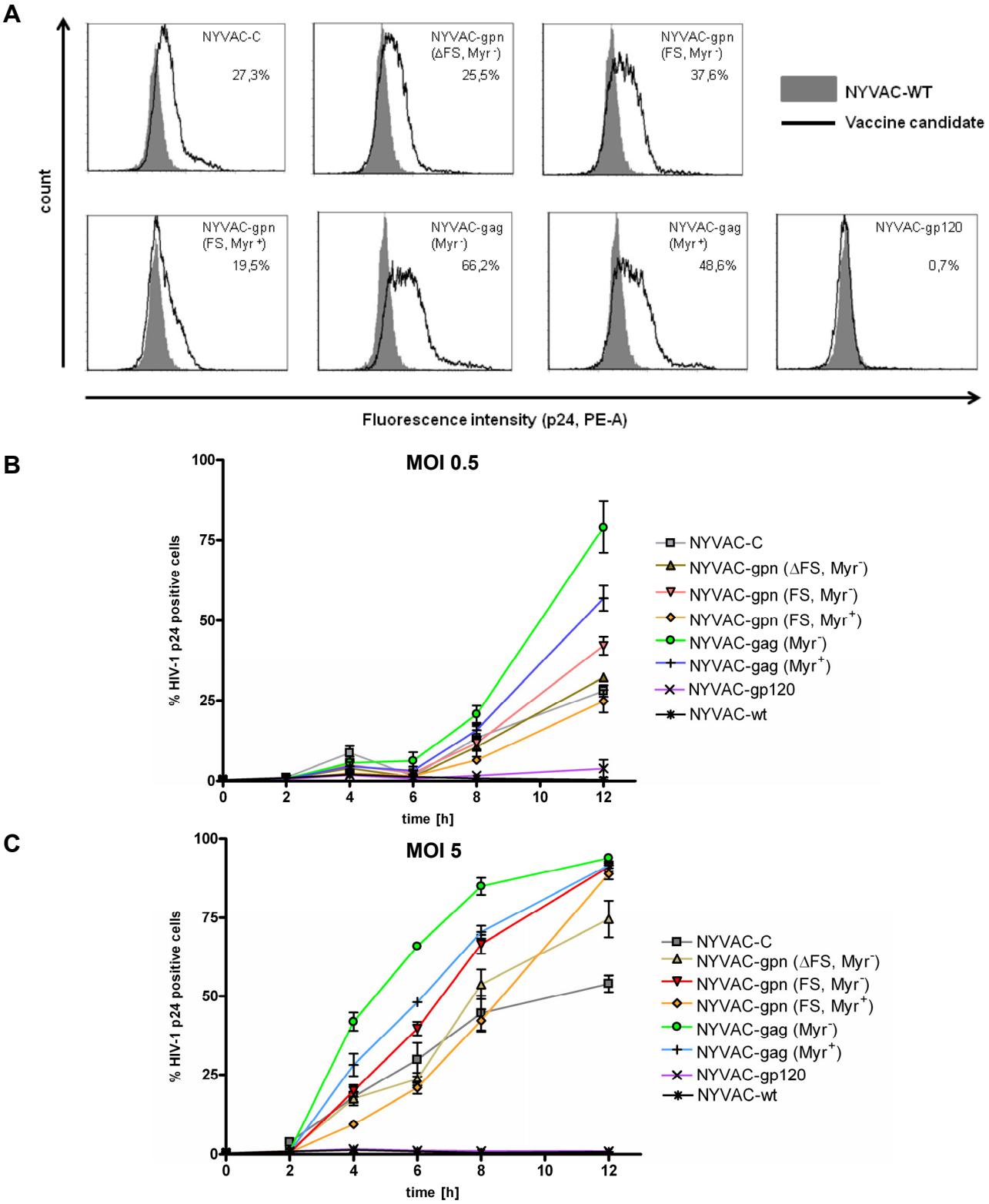


Fig. D.2 The amount of HIV-1 p24 expressing HeLa cells is strongly dependent on the vaccine candidate concentration (Page 44)

$5x10^4$ HeLa cells were infected by the indicated vaccine candidates, incubated for varying time periods and analyzed for intracellular p24 expression by FACS analysis. *A*. Exemplary histogram illustration of p24 expression in HeLa cells. The cells were infected at MOI 5 and incubated for 6 h. For comparison, the histogram of NYVAC-WT treated cells was overlaid with histograms of cells infected with the different vaccine variants. *B*. and *C*. Illustration of the time dependency of p24 expression in HeLa cells infected by vaccine variants at *B*. MOI 0.5 and *C*. MOI 5

D.2.1.1 Size-reduced immunogens induce enhanced expression in HeLa cells

To circumvent epitope competition and cytotoxic effects induced by gp120, vaccine candidates not containing gp120 and PolNef were developed. Additionally, it was supposed that size-reduction would enhance antigen expression. Compared to NYVAC-C, the size-reduced antigens were suggested to elicit an increased antigen expression. Therefore, the intracellular Gag amount was quantified by analyzing the mean fluorescence intensity.

In early infection stages, only slight differences in p24 concentration were observed between the vaccine candidates NYVAC-C, NYVAC-gpn (ΔFS, Myr$^-$) and NYVAC-gag (Myr$^-$) (see Fig. D.3). As expected, deletion of gp120 and PolNef results in an increased p24 antigen expression. Since the poxviral replication occurs within 8 hpi to 12 hpi, enhanced p24 levels detected at later timepoints of infection give a hint that virus replication occurs. Nevertheless, virus replication was detected to be ineffective in NYVAC-C infected cells (data not shown). Thus, by gp120 deletion an increased virus production rate can be achieved resulting in a 2-3 fold increased p24 amount.

By further deletion of PolNef sequences, the intracellular Gag p24 level can be escalated remarkably. Compared to NYVAC-gpn (ΔFS, Myr$^-$) infected cells, NYVAC-gag (Myr$^-$) elicits one order of magnitude increased antigen expression 12 h post infection. In summary, size reduction as well as elimination of gp120 resulted in a strongly enhanced Gag expression.

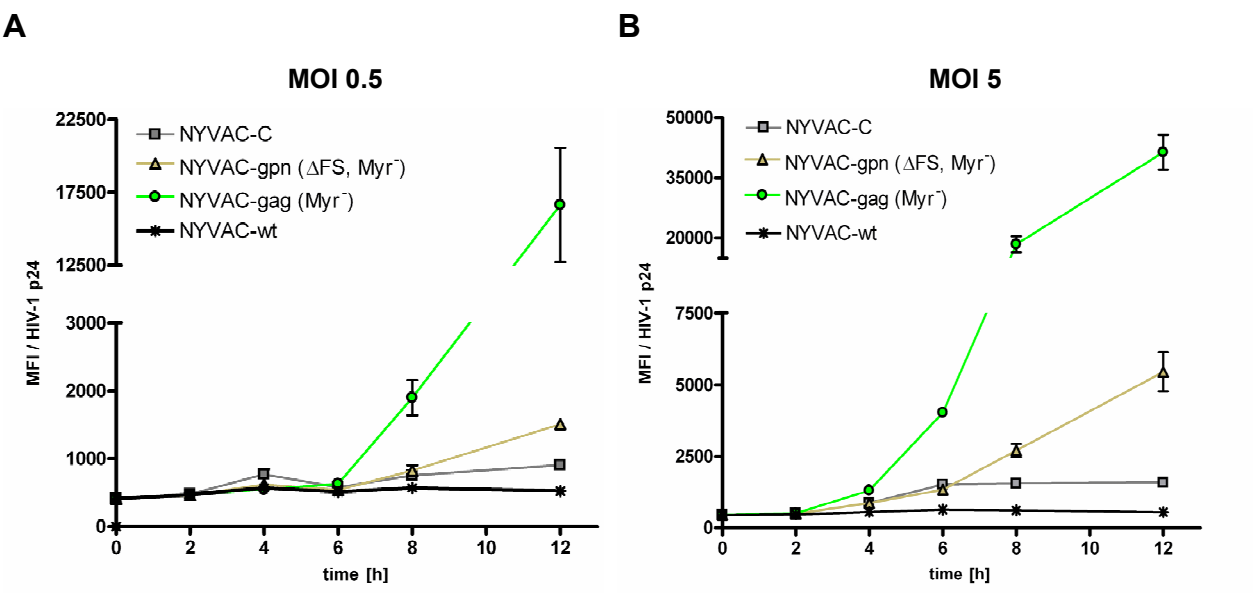


Fig. D.3 Deletion of HIV-1 gp120 and PolNef increases p24 expression after one NYVAC replication cycle

To determine the influence of epitope minimization on immunogen expression, 5x10^4 HeLa cells were infected either by NYVAC-C or NYVAC-gpn (ΔFS, Myr$^-$) and analyzed at the indicated times post infection for intracellular p24 expression by FACS analysis. Illustration of the time dependency of p24 expression in HeLa cells infected by vaccine variants at *A*. MOI = 0.5 and *B*. MOI = 5

D.2.1.2 The insertion of the natural frameshift in the GPN polyprotein results in increased HIV-1 Gag expression

To achieve enhanced Gag expression without affecting epitope presence, a ribosomal frame shift was introduced into the GPN polyprotein. This modification was described to result in Gag:GPN expression in a ratio of 95:5 [250]. To observe the influence of the naturally occurring ribosomal frame shift on antigen expression, the intracellular p24 amount in vaccine candidate infected HeLa cells was analyzed.

When comparing NYVAC-gpn (ΔFS, Myr$^-$) and NYVAC-gpn (FS, Myr$^-$), a 3 to 4-fold increased p24 steady-state level was observed for the immunogens with frame shift 12 hpi (see Fig. D.4). As expected, the ribosomal frameshift induces a strong increase in p24 amount. Thus, introduction of a -1 frame shift by reconstitution of the initial codon usage at the amino acid positions 1293 to 1345, the Gag expression was enhanced without affecting epitope presence.

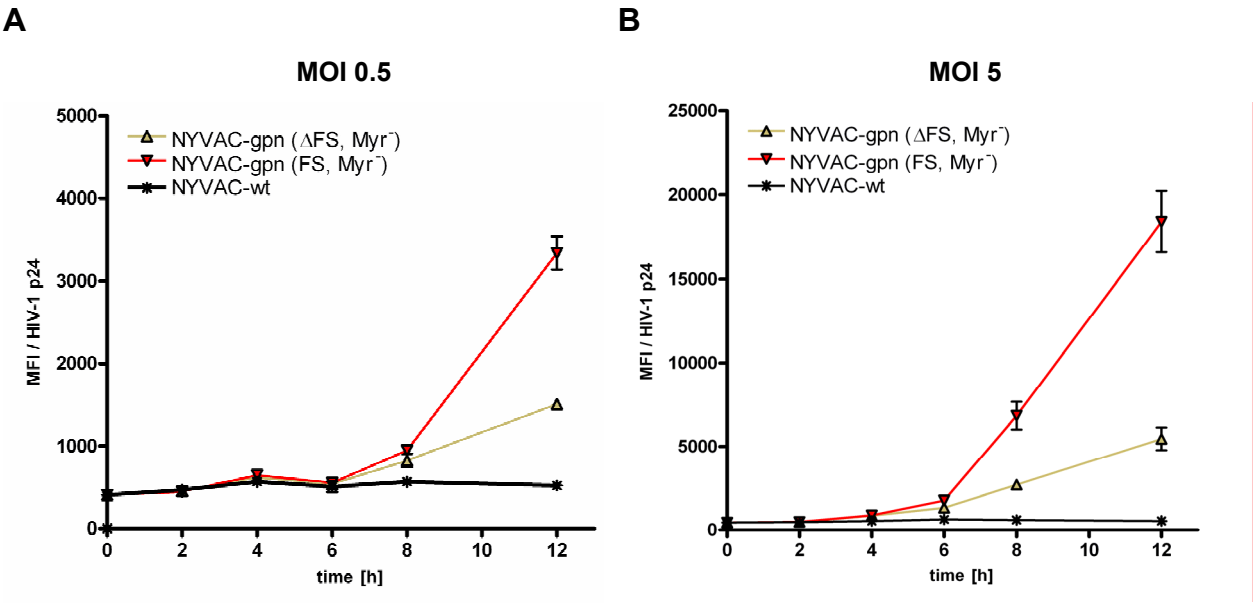


Fig. D.4 A frame shift insertion in the GPN polyprotein leads to increased intracellular p24 amounts

To determine the influence of a ribosomal frame shift on immunogen expression, 5x10^4 HeLa cells were infected by either NYVAC-gpn (ΔFS, Myr$^-$) or NYVAC-gpn (FS, Myr$^-$) and analyzed at the indicated times post infection for intracellular p24 expression by FACS analysis. Illustration of the time dependency of p24 expression in HeLa cells infected by vaccine variants at *A*. MOI = 0.5 and *B*. MOI = 5

D.2.1.3 Myristoylation of Gag diminishes the intracellular Gag steady-state levels due to budding effects

For efficient cross presentation, the immunogen has to be available extracellularly in sufficient amounts. The ability of Gag to form VLP was described to facilitate cross presentation [234,235]. Due to a translational block of late poxviral genes in APC, cross presentation is necessary when using NYVAC as vaccine delivery system. VLP budding requires efficient membrane anchoring. Therefore, HIV-1 Gag has to be myristoylated posttranslationally. An altered myristoylation site was described to inhibit VLP formation efficiently [19].

To observe the impact of budding on the intracellular Gag amount, p24 was quantified in vaccine candidate infected HeLa cells (see Fig. D.5). As detected in Western-blot analysis (see Fig. D.1) when comparing NYVAC-gag (Myr^-) with NYVAC-gag (Myr^+), a 5-fold increase of intracellular p24 concentration was observed 12 h post infection. NYVAC-gpn (FS, Myr^+) infected cells contain a 3-fold decreased Gag amount compared to NYVAC-gpn (FS, Myr^-) infected cells. Hence, inhibition of p55 VLP formation resulted in an intracellular Gag accumulation. Again, the MOI strongly influenced the transgene expression levels. A 10-fold increased virus multiplicity induced an almost 3-fold increased Gag concentration. Nevertheless, antigen expression did not increase in a linear manner compared to the applied virus concentration. Thus, a limitation of transgene expression is suggested.

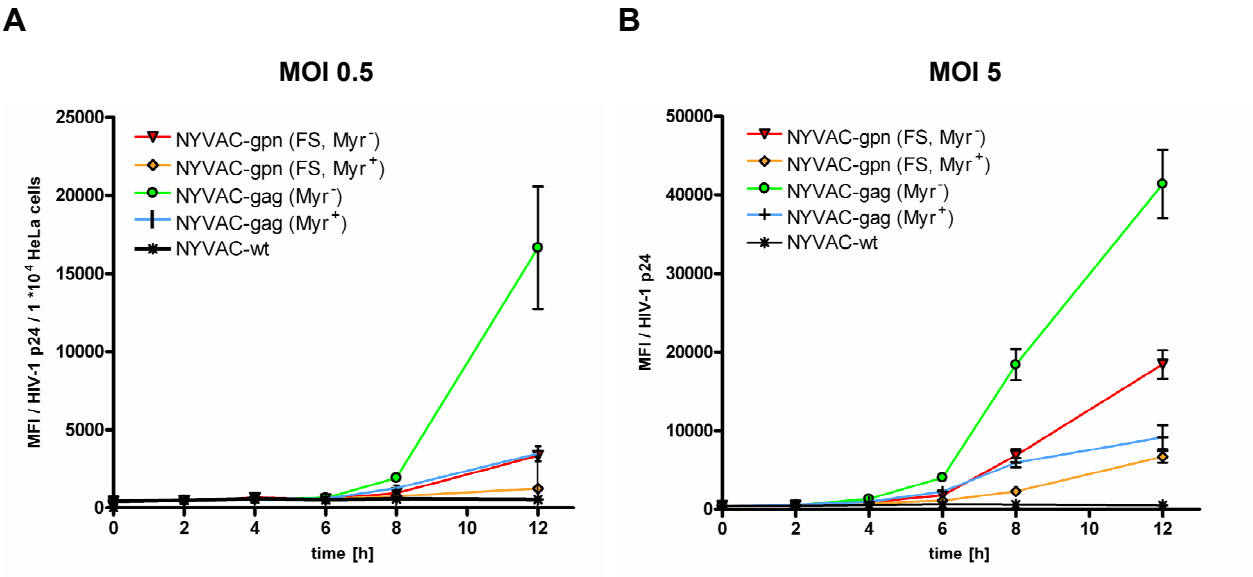


Fig. D.5 VLP formation evoked by HIV-1 Gag myristoylation leads to decreased intracellular p24 amounts

To determine the influence of VLP budding on immunogen expression, $5x10^4$ HeLa cells were infected by NYVAC coding either for HIV-1 antigens with intact or mutated Gag myristoylation site. After incubation, the cells were analyzed at the indicated times post infection for intracellular p24 expression by FACS analysis. Illustration of the time dependency of p24 expression in HeLa cells infected by vaccine variants at *A*. MOI = 0.5 and *B*. MOI = 5

D.2.2 HIV-1 Gag based immunogens could be detected in low amounts in NYVAC infected mdDC

Although antigen cross presentation is described to be sufficient for the generation of CTL based immunity induced by vaccination, direct presentation could complement or enhance vaccine induced immunity [121]. Due to their localization in epidermal tissues, Langerhans cells (LDC) represent an APC population that is a key player in generating vaccine induced immunity. Vaccines based on viral vector systems can enter the APCs' cytoplasm either by infection mediated by receptor mediated endocytosis. Therefore, to achieve direct presentation, the antigen delivery system has to infect DC such as LDC productively. Poxviral vector systems were described to infect APC only slightly resulting in poor antigen expression (see B.3). Nevertheless, the vaccine candidates were observed whether they infect mdDC directly.

To analyze the immunogen expression characteristics in APCs, mdDCs cells were infected with different vaccine candidates by two different concentrations. After incubation for varying time periods the cells were stained with an anti-p24 antibody. The target cells were analyzed to express the corresponding immunogens by FACS analysis.

NYVAC uptake as well as an efficient antigen expression could be detected in mdDC (see Fig. D.6). As described for HeLa cells (see D.2.1) a strong influence of the virus concentration on the ratio of $p24^+$ cells was observed. HIV-1 Gag was analyzed to be expressed within few hours post infection. At low MOI infections, up to 18% p24 positive cells were quantified (see Fig. D.6 *B*). In contrast to transduced HeLa cells, no decline in transgene expression was observed for most of the vaccine candidates.

Again, VLP formation induced a decline in the number of $p24^+$ cells. Notably, the size-reduced vaccine candidate NYVAC-gag (Myr^-) did not induce increased p24 expression compared to NYVAC-gpn (ΔFS, Myr^-) and NYVAC-gpn (FS, Myr^-).

Infection with high virus multiplicities resulted in increased intracellular Gag concentrations. Almost 40 % of the employed cells were detected to contain p24 (see Fig. D.6 *C*). Expression was shown to peak at 8 hpi.

Consistent with the results obtained for HeLa cells, the NYVAC-gag (Myr^-) vaccine candidate coding for the size-reduced immunogen induced strongest antigen expression. Immunogen enriched vaccine candidates such as NYVAC-gpn (ΔFS, Myr^-) elicited low Gag content in mdDCs. Infection with the gp120 containing NYVAC-C variant led to a complete loss of Gag expression 8 hpi independently of the applied MOI.

Compared to infection of HeLa cells, immunogen expression in mdDC was shown to be less productive. Especially at later time points, a lower number of Gag containing cells were detected. These results are consistent with published data, which show that NYVAC replication is limited in mdDC (see B.1.3.4).

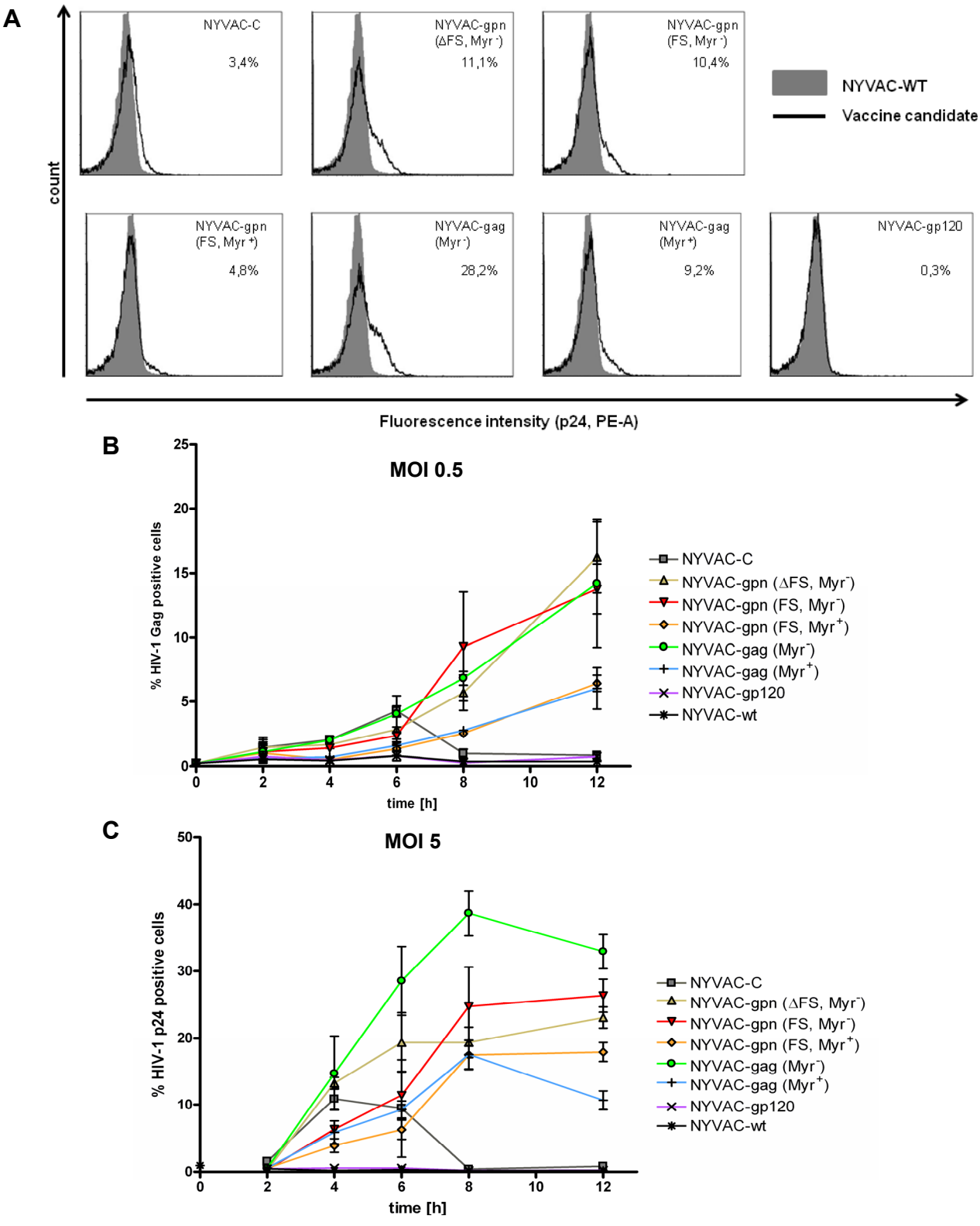


Fig. D.6 The expression of HIV-1 p24 in NYVAC infected mdDCs cells is strongly dependent on the MOI

$5x10^4$ mdDCs were infected by the indicated vaccine candidates, incubated for varying time periods and analyzed for intracellular p24 expression by FACS analysis. *A*. Exemplary histogram illustration of p24 expression in mdDC. The cells were infected at MOI 5 and incubated for 6 h. For comparison, the histogram of NYVAC-WT treated cells was overlaid with histograms of cells infected with the different vaccine variants. *B*. and *C*. Illustration of the time dependency of p24 expression in mdDCs infected by vaccine variants at *B*. MOI 0.5 and *C*. MOI 5

D.2.2.1 Myristoylation of Gag diminishes the intracellular Gag amounts slightly whereas immunogen minimization has no influence on the antigen expression in mdDC

To further observe the influence of immunogen design on expression in mdDC, Gag levels were quantified in mdDC infected with different vaccine candidates. For HeLa cells it was shown that introduction of a ribosomal frame shift boosted antigen expression (see D.2.1.2) whereas VLP formation diminished the intracellular p24 amount strongly (see D.2.1.3). Since infected DCs are shown to suppress expression of late viral genes partially [79,83], only modest immunogen expression was detected. Thus, p24 detection 12 hpi is exemplary illustrated (Fig. D.7). Nevertheless, despite the long incubation period, the vaccine candidates induce only slight immunogen expression.

In contrast to vaccine candidate infected HeLa cells, mdDC infected with NYVAC-gpn (FS, Myr^-) did not express increased Gag amounts compared to NYVAC-gpn (ΔFS, Myr^-). The intracellular p24 concentration was shown not to differ between both variants (see Fig. D.7). This indicates that in mdDC, a transcriptional or translational limitation masks different immunogen expression capacities that were observed for HeLa cells.

In NYVAC-gag (Myr^-) infected mdDC, an increased p24 steady-state level was detected compared to NYVAC-gag (Myr^+) targeted cells. Certainly, in mdDC the observed differences between both vaccine candidates are less distinct than in HeLa cells. Due to limited immunogen expression in mdDC, NYVAC-gag (Myr^-) / NYVAC-gpn (FS, Myr^-) only induced a slightly increased p24 amount compared to NYVAC-gag (Myr^+) / (FS, Myr^+).

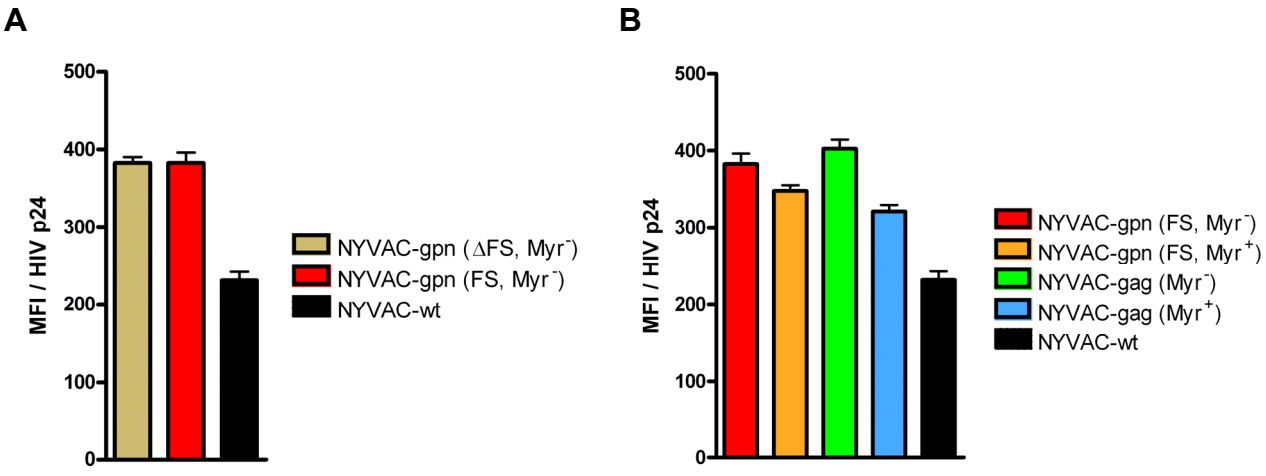


Fig. D.7 The insertion of the natural HIV frameshift in the GPN polyprotein has no effect on p24 expression while VLP budding reduces the intracellular p24 content in mdDCs slightly

$5x10^4$ mdDCs were infected by the indicated vaccine candidates at MOI 5, incubated for varying time periods and analyzed for intracellular p24 expression by FACS analysis. *A*. Illustration of the influence of frame shift reconstitution in the artificial GPN polyprotein on intracellular p24 amount in mdDCs infected with the indicated vaccine candidates. *B*. Illustration of the impact of VLP formation on the intracellular p24 amount in mdDCs infected with the indicated vaccine candidates.

D.3 NYVAC elicits a poor maturation effect of mdDC

Professional APCs such as DC are the only cell types that are able to translate innate into adaptive immune responses. APC can capture immunogens and process them into peptides, which are presented on the cell surface. Antigen presentation can initiate cellular immune responses. For efficient T cell stimulation, naïve APC alter their phenotype by upregulation of distinct molecules. This maturation process comprises upregulation of (i) antigen presenting HLA complexes, (ii) co-stimulatory molecules such as CD80 and CD86 and (iii) chemokine receptors that mediate migration to lymph nodes (see B.2.2.1). Maturation can be driven by bacterial antigens (e.g. LPS), inflammatory cytokines, ligands of surface receptors (e.g. CD40), or viral products. Although NYVAC was shown to induce only incomplete DC maturation due to immune escape mechanisms, efficient T cell priming was observed in vaccination studies (see B.3).

Since DC maturation is essential for T cell stimulation, the vaccine candidates were analyzed concerning their DC activation capacity. Besides the stimulation induced by the viral vector backbone, the immunogen's influence on DC maturation was determined. For detailed analysis of the DC activation potential, the vaccine candidate-driven upregulation of several maturation markers was observed. Besides the antigen presenting HLA class-II protein HLA-DR, upregulation of the co-stimulatory molecules CD80 and CD86 was evaluated. For analysis, mdDC were infected with the vaccine candidates by two different MOI. After several time periods the surface markers were detected by specific antibodies. The cells were analyzed to express the corresponding maturation markers by FACS analysis.

As described by Guerra *et al*. [79], NYVAC infected mdDC were shown to maturate incompletely (see Fig. D.8). The co-stimulatory molecules CD80 and CD86 (also known as B7-1 and B7-2) were not upregulated during the maturation process. Remarkably, the level of CD80 and CD86 positive cells declined between 24 hpi and 48 hpi, which was not observed for LPS stimulated cells. Virus multiplicity did not influence mdDC maturation (see Fig. D.8 B-E).

The antigen presenting HLA class-II molecule HLA-DR was shown to be upregulated in NYVAC infected DC (see Fig. D.8 *F* and *G*). HLA-DR upregulation was observed 12 hpi, whereas the maximal surface concentration was achieved 24 h post infection. As monitored for CD80 and CD86, the number of HLA-DR positive cells declined 48 h after NYVAC infection. At increased NYVAC multiplicities, upregulation of HLA-DR was enhanced slightly.

Altogether, no significant differences between the varying vaccine candidates were detected 24 h after DC infection. A decreased maturation marker amount on the cell surface 48 h post infection was detected for all candidate vaccines except NYVAC-gpn (ΔFS, Myr$^-$). Thus, in the case of the developed vaccine candidates the immunogens play only a minor role in inducing DC maturation.

A

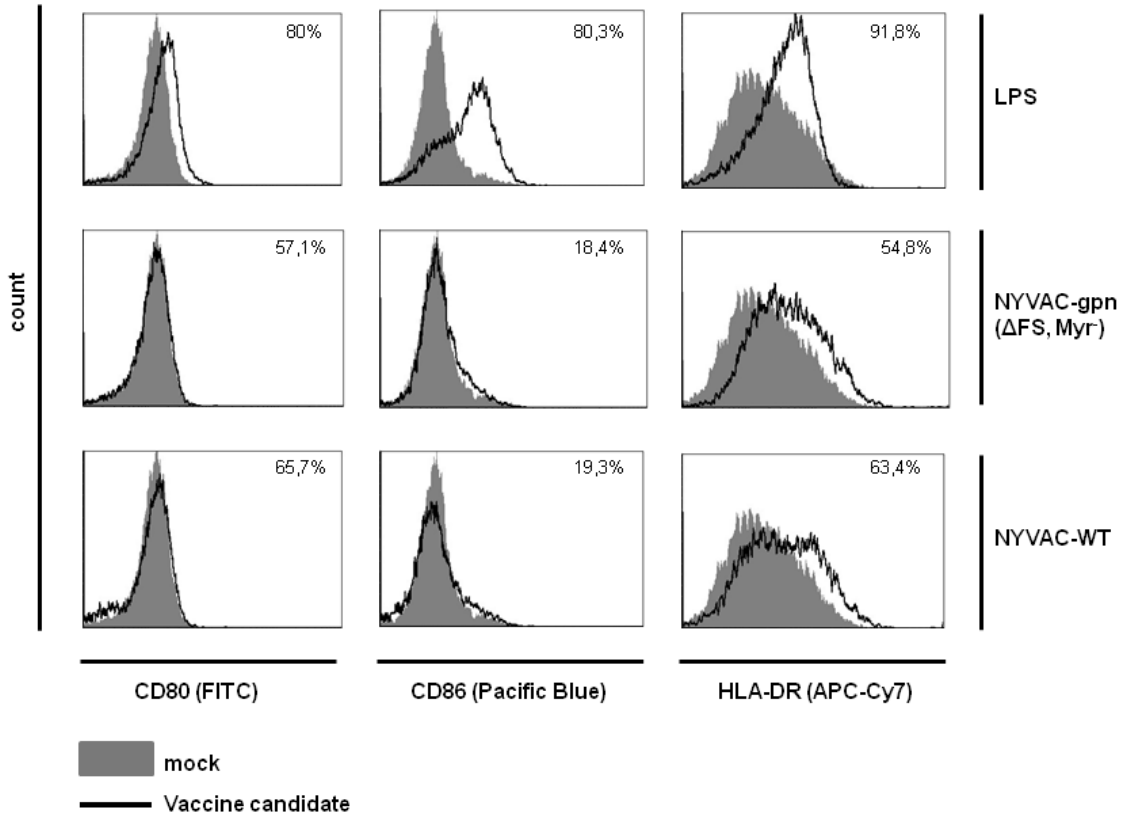


Fig. 8 is continued on page 53

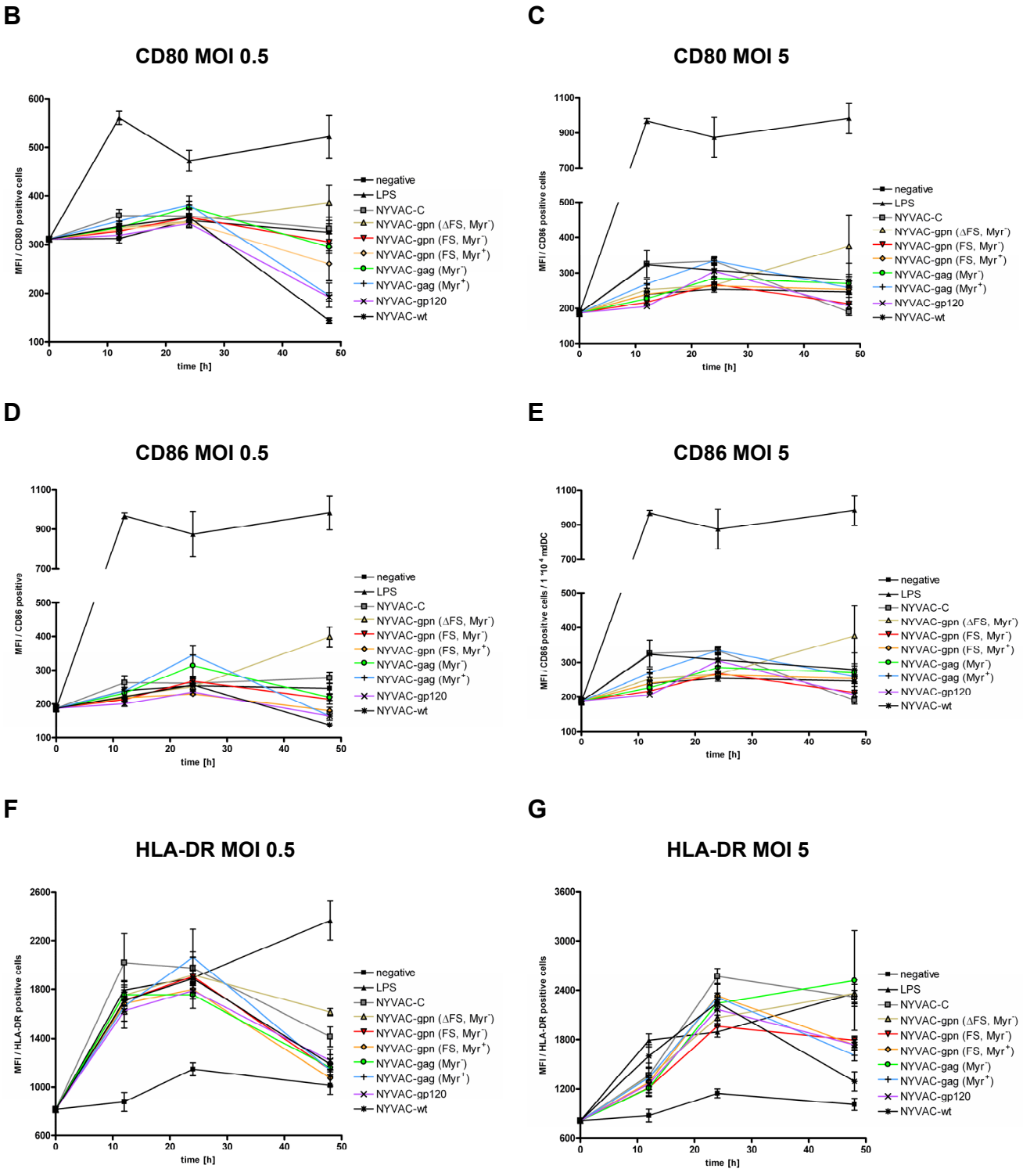


Fig. D.8 NYVAC but not HIV-1 derived immunogens induce partial mdDC maturation

$5x10^4$ mdDCs were infected by different vaccine candidates at the indicated MOI, incubated for different time periods and analyzed for maturation marker expression by FACS analysis *A*. Exemplary histogram illustration of quantifying maturation markers on mdDCs. The cells were infected at MOI = 5 and incubated for 24 h. For comparison, the histogram of untreated mdDCs was overlaid with histograms of cells infected with the different vaccine candidates. *B.* to *G*, Determination of the time dependency of maturation marker presentation on mdDCs infected with the different vaccine candidates at MOI 0.5 or 5.

D.4 The HIV-1 Gag-derived peptide GL9 can effectively be presented directly on vaccine-infected mdDC

Direct presentation of antigen derived peptides is required for the generation of immune responses directed against intracellular pathogens or antigens. Therefore, an efficient vaccine is required to induce both transgene expression as well as maturation of DC present at vaccination sites. The engineered new generation antigens were analyzed for their ability to be processed into peptides that subsequently are presented on the APC surface by direct presentation. Additionally, the vaccine induced mdDC maturation was demonstrated to be sufficient for T cell restimulation. For restimulation assays, a Gag specific $CD8^+$ cytotoxic T cell clone isolated from an HIV infected donor was utilized. The CTL clone expresses a TCR that specifically binds to the HIV-1 Gag p24-derived peptide GPGHKARVL (GL9) in complex with an HLA-B*07 molecule. Recognition of a sufficient amount of GL9-HLA-B*07 pMHC complexes as well as co-stimulatory molecules induces CTL restimulation resulting in IFN-γ release. By inhibiting ER-mediated export with Brefeldin A, IFN-γ expression can be detected by ICS staining and FACS analysis. mdDC were generated from monocytes derived from HLA-B*07 positive donors. To analyze the immunogens' capacity to be presented on APC, the influences of (i) incubation time and (ii) virus concentration on direct presentation were evaluated.

(i) To determine the optimal timepoint between peptide presentation and maturation, the co-culture of NYVAC-infected mdDC with GL9-specific CTL was started at varying timepoints.

(ii) The influence of virus multiplicity on direct presentation was analyzed at the optimal cocultivation conditions defined previously.

The experimental layout of the direct presentation assay is illustrated in Fig. D.9.

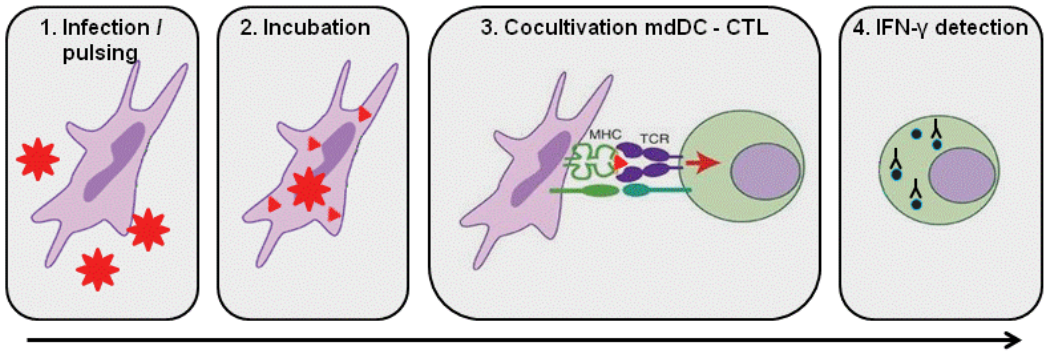


Fig. D.9 Experimental layout of the direct presentation assays

For direct presentation analysis, mdDC derived from a HLA-B*07$^+$ donor were infected with the vaccine candidates at (i) defined or (ii) varying virus multiplicities. After incubation for (i) varying time points or (ii) 6 h, the mdDC were cocultivated with an equal amount of a GL9-specific T cell clone. After an incubation period of 12 h, the T cells were evaluated to express IFN-γ by FACS analysis.

For FACS analysis, T lymphocytes were detected by analysis of size (FSC) and inner complexity (SSC, see Fig. D.10 *A*). CTL were identified by staining with a CD8 specific antibody (see Fig. D.10 *B*). $CD8^+$ cells were analyzed for IFN-γ expression by ICS. Peptide-pulsed mdDC represent the positive control (see Fig. D.10 *B*).

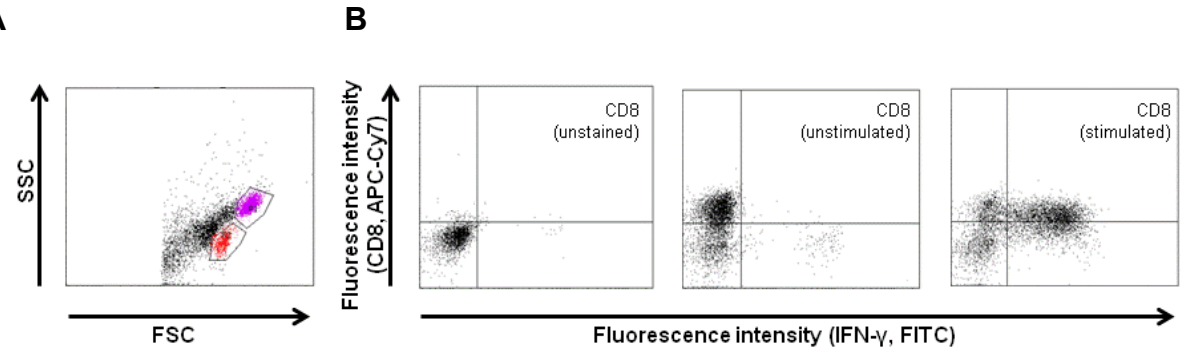


Fig. D.10 Population determination in FACS analysis of direct presentation assays

A. Illustration of the population distribution between CTLs and mdDCs in a direct presentation assay. The cocultivated HLA-B*07 mdDC and CTL were gated by analysis of cell volume (FSC) and inner complexity (SSC) at which the gate coloured in red corresponds to T lymphocytes. *B*, T lymphocytes were determined to express CD8 in a surface staining. In addition, an ICS of IFN-γ was performed. For calibration purposes, an unstained control was analyzed. To control proper gating, unstimulated as well as GL9 peptide stimulated CTLs were detected.

D.4.1 Direct presentation of the HIV-1 Gag derived GL9 peptide is strongly influenced by the immunogen characteristics

As shown, transgene expression and maturation of vaccine candidate infected mdDC was induced modestly (see 2.3.2 and D.4). Despite those low levels of intracellularly detected antigens, direct presentation of Gag-derived peptides was analyzed in an *in vitro* asay.

Surprisingly, despite low expression levels in infected mdDC, the vaccine variants coding for the GPN polyprotein were shown to induce the most extensive GL9 CTL restimulation (see Fig. D.11 and D.12 *A* and *B*). Although NYVAC-gpn (ΔFS, Myr^-) induced more extensive immunogen expression in mdDC compared to NYVAC-C (see Fig. D.1) both vaccine candidates stimulated high numbers of Gag specific CTLs. At low MOI infections (MOI 0.5) 14-18% of the applied T lymphocytes became activated (see Fig. D.11 *B*). Infection with an increased multiplicity (MOI 5), led to a CTL restimulation rate of 37 - 42% (see Fig. D.11 *C*).

Interestingly, peptide presentation induced by the new generation immunogens was reduced strongly in spite of increased expression levels. Compared to GPN (FS^-), GL9 direct presentation of the GPN (FS^+) immunogen was decreased 5 to 10-fold dependent on the MOI (see D.12 *A* and *B*).

When infecting with high virus multiplicities, elimination of PN recovered the peptide presentation to some extent. NYVAC-gag (Myr^-) and NYVAC-gag (Myr^+) infected mdDC were capable of restimulating 23 and 15 % of the applied T lymphocytes, respectively (see Fig. D.12 *C* and *D*). As shown, VLP formation diminishes intracellular Gag concentration (see D.3.2.1 and D.3.1.3). Consequently, budding competence results in reduced peptide presentation in Gag (Myr^+) transgenic cells. Slight differences can be observed between NYVAC-gpn (FS, Myr^-) and NYVAC-gpn (FS, Myr^+) infected mdDC (see Fig. D.12 *C* and *D*).

Remarkably, CTL stimulation by direct presentation was indeed achieved subsequently after NYVAC infection. This suggests that (i) NYVAC uptake, (ii) transgene expression and proteasomal digestion as well as (iii) presentation occurred within a minimal interval of 4 hpi. At low MOI infections, no decline in antigen presentation was observed (see Fig. D.11 *B*). This indicates, that the GL9 peptide was presented over an at least 12 h time-period as measured by the CTL restimulation capacity. When infecting with high virus amounts, the most extensive CTL restimulation was detected 6 h post infection (see Fig. D.11 *C*). Summing up, the new generation vaccine candidates require administration at high MOI, which results in necrosis of the target cells. Necrotic cells can stimulate immune responses via cross presentation but do not participate in direct presentation [258].

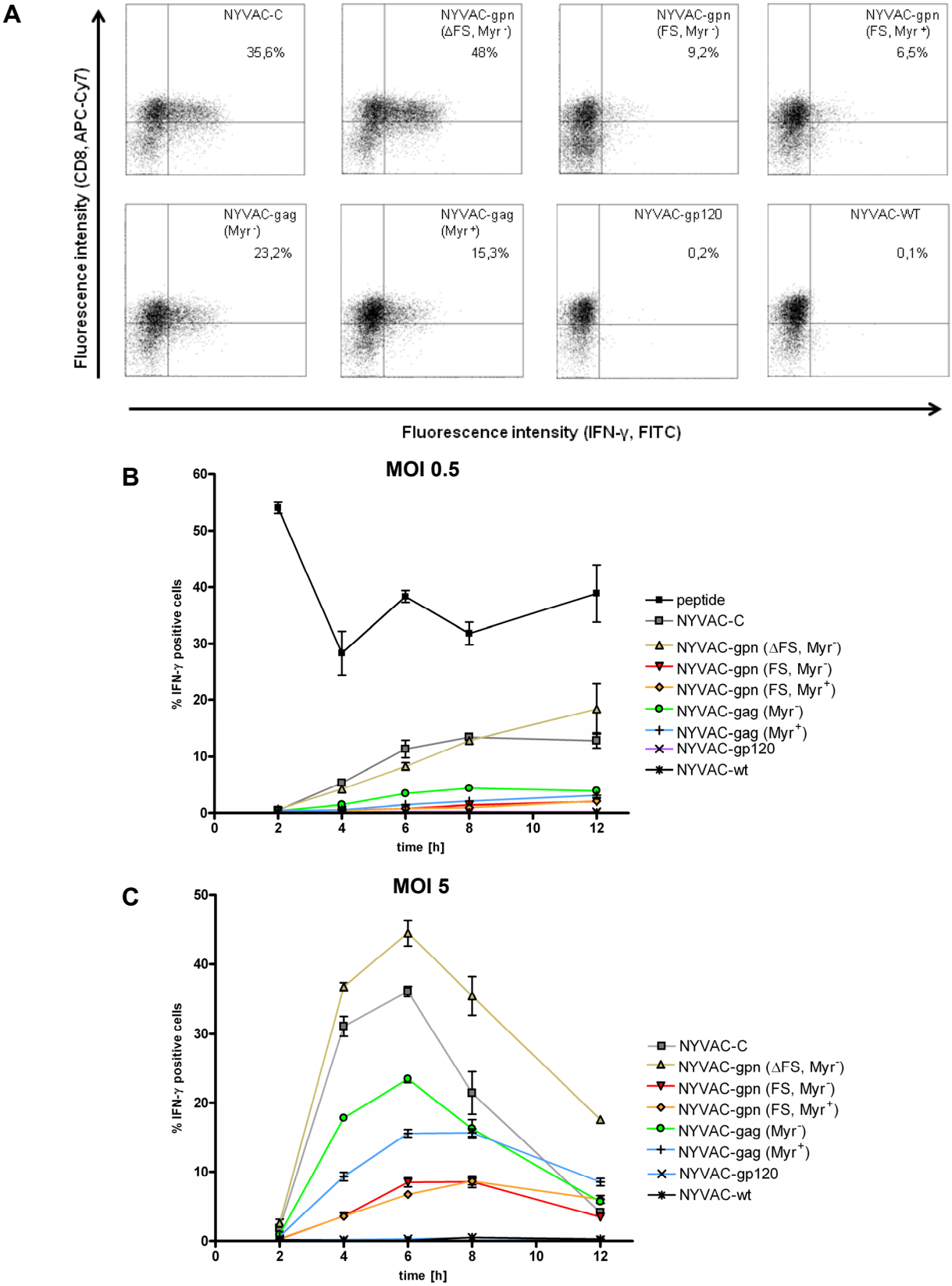


Fig. D.11 GL9 presentation by NYVAC infected mdDCs is strongly dependent on immunogen characteristics and the applied virus amount

$5x10^4$ mdDC were infected with different vaccine candidates, incubated for the indicated periods and cocultivated with GL9 CTLs. $CD8^+$ CTLs were investigated for IFN-γ expression by FACS analysis. *A*. Exemplary dot plot illustration of GL9 CTL restimulation by mdDCs infected with the indicated vaccine candidates. The cells were infected at MOI 5 and incubated for 6 h. T lymphocytes were analyzed for expression of both CD8 and IFN-γ. *B*. and *C*. Illustration of the time dependency of CTL restimulation by direct presentation. mdDCs were infected with the indicated vaccine candidates at *B*. MOI 0.5 and *C*. MOI 5.

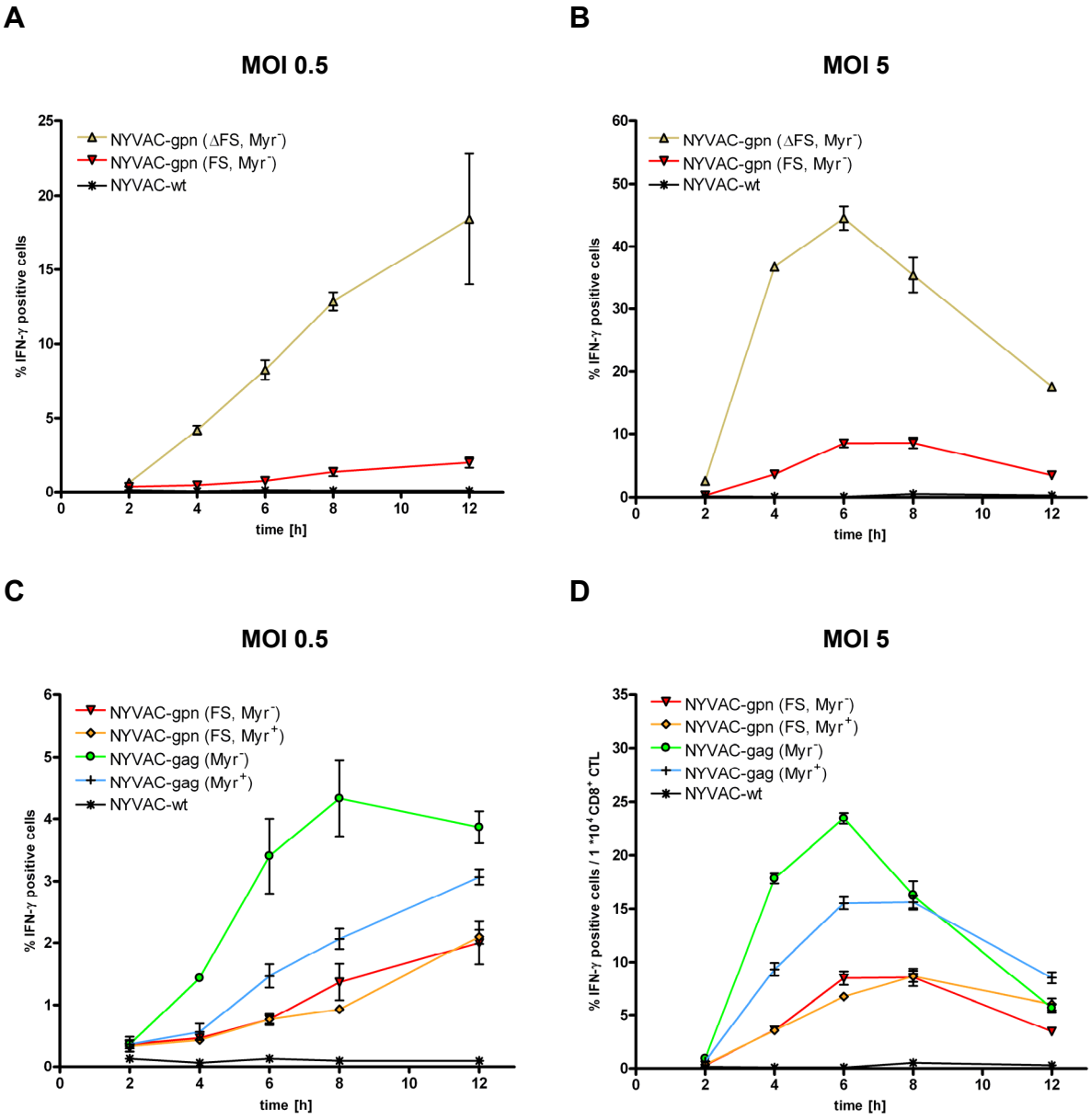


Fig. D.12 Frame shift insertion as well as VLP formation influence direct presentation of the Gag-derived GL9 peptide

$5x10^4$ mdDC were infected with the indicated vaccine candidates, incubated for varying periods and cocultivated with GL9 CTLs. $CD8^+$ CTLs were investigated for IFN-γ expression by FACS analysis *A*. and *B*. Illustration of the influence of frame shift insertion into the artificial GPN polyprotein on GL9 direct presentation on mdDCs. mdDC were infected by vaccine variants at *A*. MOI 0.5 and *B*. MOI 5. *C*. and *D*. Illustration of the influence of VLP formation on on GL9 direct presentation on mdDCs. The cells were infected with vaccine variants at *C*. MOI 0.5 and *D*. MOI 5.

D.4.2 The direct presentation efficiency is strongly dependent on the NYVAC concentration

In direct presentation, a strong dependency on virus multiplicity can be observed (see D.4.1). The ability of some antigens to be directly presented on mdDC strongly depends on the virus amount (see Fig. D.10). To analyze the influence of the NYVAC concentrations further, mdDC were infected with a set of virus multiplicities. Direct presentation was determined 6 h post infection by co-cultivation of a GL9 specific T cell clone.

Again, the most extensive GL9 CTL restimulation was induced by the artificial GPN polyprotein (see Fig. D.13). mdDC infection with NYVAC-gpn (ΔFS, Myr^-) and NYVAC-C at MOI 0.1 was sufficient to mediate activation of considerable CTL numbers. For both vaccines, maximal CTL restimulation was observed when infecting mdDC with MOI > 1. The CTL activation potential of the remaining vaccine candidates was increased in an almost linear manner again being highest at MOI 1 to 2. Compared to NYVAC-gpn (ΔFS, Myr^-) and NYVAC-C, GL9 presentation was observed to be less extensive despite enhanced immunogen expression.

When exceeding virus multiplicities of 10, a strong decline in direct presentation was detected for all vaccine candidates. Infections with high NYVAC concentrations induce necrosis. As described, necrotic cells can stimulate immune responses via cross presentation but do not participate in direct presentation [258]. This indicates that the vaccines have to be administered in well-defined and accurate amounts. Low virus multiplicities are not sufficient to activate large numbers of CTL whereas over-concentration aborts direct presentation of the GL9 peptide.

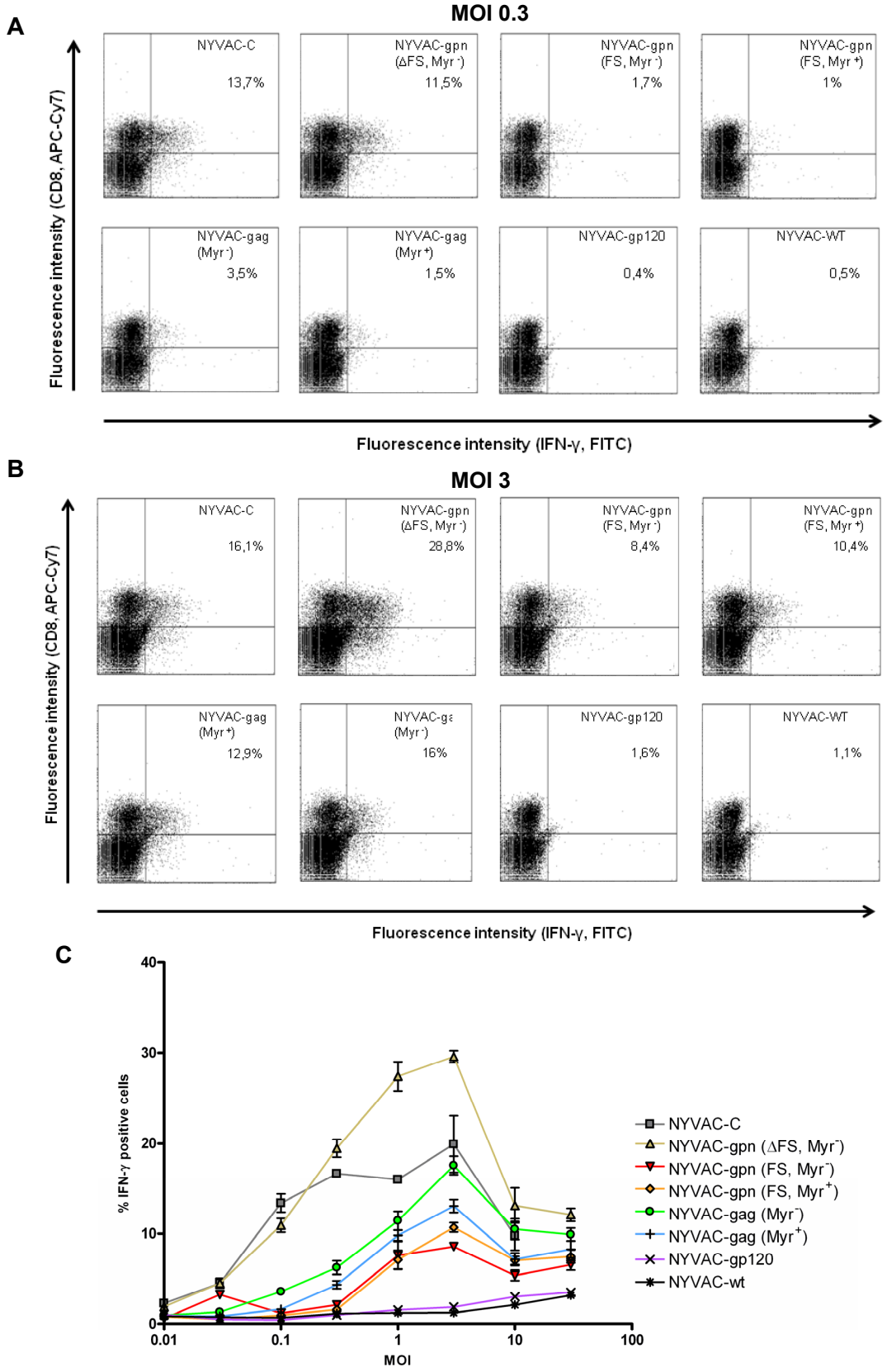


Fig. D.13: Efficient GL9 direct presentation requires a well balanced NYVAC multiplicity

$5x10^4$ mdDC were infected with the indicted vaccine candidates at varying MOI, incubated for 6 h and cocultivated with an equal amount of GL9 CTLs. $CD8^+$ CTLs were analyzed for IFN-γ expression by FACS analysis. *A*. and *B*. Exemplary dot plot illustration of GL9 CTL restimulation by vaccine infected mdDC. The cells were infected with the indicated vaccine candidates and incubated for 6 h. T lymphocytes were analyzed to express both CD8 and IFN-γ. mdDC were infected by vaccine variants at *A*. MOI 0.3 and *B*. MOI 3. *C*. Illustration of the restimulation of $CD8^+$ cells dependent on virus multiplicity in a direct presentation assay.

D.5 The HIV-1 Gag-derived GL9 peptide can be presented on NYVAC infected mdDC by cross presentation

When administering vaccines by intramuscular injection, mainly cells other than APC such as fibroblast or epithelial cells are targeted by the vaccine. Professional APC can take up cells, cell debris and extracellular antigens. Therefore, proteins expressed in tissue cells represent a source of immunogens allowing T lymphocyte activation. Extracellular proteins are endocytosed and degraded. Peptides derived from extracellular proteins are mainly not targeted to MHC class-I compartments but are presented on MHC class-II molecules [124,125]. By cross presentation, these peptides can be presented on MHC-class-I molecules. The ability to be cross presented on MHC class-I molecules strongly depend on the antigen design (see B.2.1). In generating T cell immunity, cross presentation can support the direct presentation pathway. For poxviral vector systems, cross presentation was described to be the major mode of inducing CTL responses [142].
To analyze the new generation antigen's cross presentation capacity, vaccine candidate infected cells were evaluated for induction peptide presentation on mdDC. As a model system, the human epithelial tumor cell line HeLa was utilized for cross presentation assays. NYVAC infected HeLa cells were shown to express the Gag-derived transgenes efficiently (see D.3.1). As described for direct presentation assays, the HIV-1 GL9 specific CTL clone was applied for the detection of cross presentation. Again, the (i) time dependency of cross presentation and (ii) the influence of the virus concentration were evaluated. To avoid direct mdDC infection, viruses loosely attached on HeLa cells or present in the supernatant were eliminated by multiple washing steps. By washing the virus concentration in the supernatant was reduced 200-fold (data not shown). Since NYVAC was found to replicate in HeLa cells, mdDC infection with virus progenitors derived from infected cells has to be excluded. Therefore, the cross presentation assay was developed not to exceed a duration of 8 h until CTL cocultivation was initiated.

(i) To determine the optimal timepoint for cocultivation of NYVAC-infected HeLa cells with mdDC. the coculture was initiated at varying timepoints.
(ii) The influence of virus multiplicity on cross presentation was analyzed adopting the optimal cocultivation conditions defined previously.

The experimental layout of the cross presentation assay is illustrated in Fig. D.14.

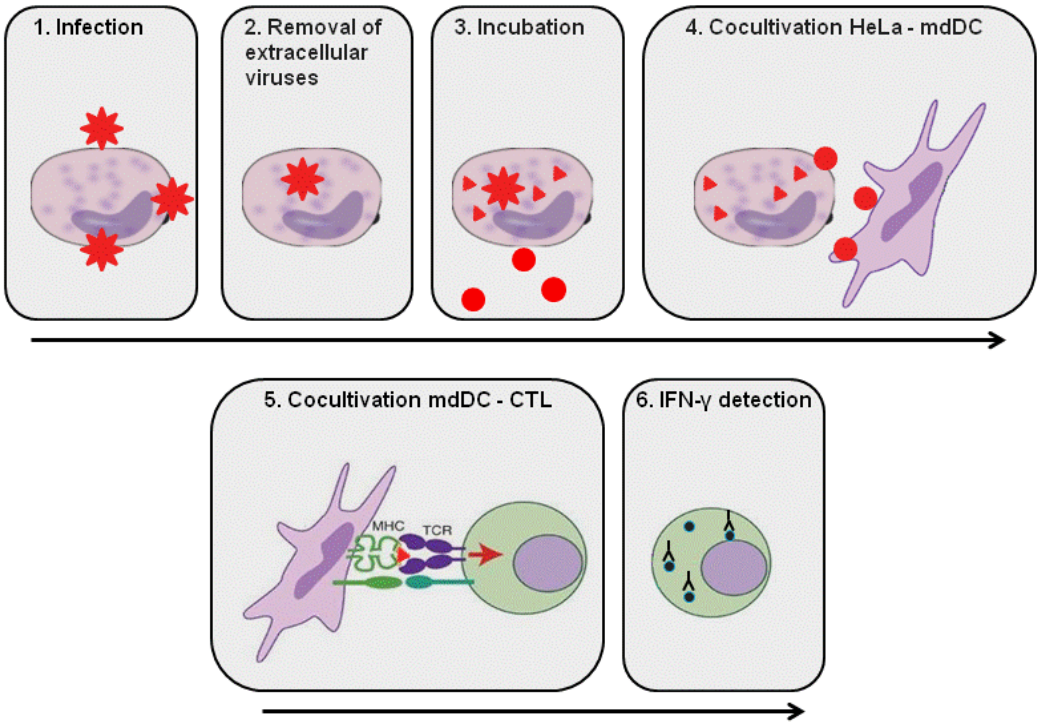


Fig. D.14 Experimental layout of cross presentation assays

For cross presentation analysis, HeLa cells were infected with the vaccine candidates by (i) MOI 5 or (ii) varying virus multiplicities. After removal of extracellular viruses by 5 washing steps 1 h post infection cells were incubated for 3 h. After incubation the HeLa cells were cocultivated with mdDC derived from a HLA-B*07$^+$ donor. After incubation for (i) varying time points or (ii) 4 h, the mdDC were cocultivated with an equal amount of a GL9-specific T cell clone. After an incubation period of 12 h, the T cells were evaluated to express IFN-γ by FACS analysis.

GL9-CTL restimulation was determined by FACS analysis. T lymphocytes were detected by analysis of size (FSC) and inner complexity (SSC, see Fig. D.10 *A*). CTL were identified by staining with an antibody directed against the CD8 receptor (see Fig. D.10 *B*). CD8$^+$ cells were analyzed for IFN-γ expression by ICS. Peptide-pulsed mdDC represent the positive control (see Fig. D.10 *B*).

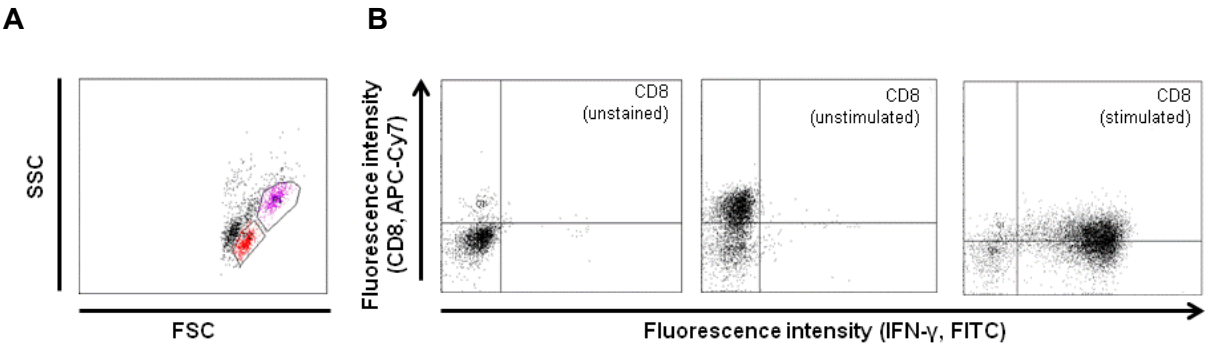


Fig. D.15 Population determination in FACS analysis of cross presentation assays

A. Illustration of the population distribution between CTLs and mdDCs in a cross presentation assay. The cocultivated HeLa cells, HLA-B*07 mdDC and CTL were gated by analysis of cell volume (FSC) and inner complexity (SSC) at which the gate coloured in red corresponds to T lymphocytes. *B*, T lymphocytes were determined to express CD8 in a surface staining. In addition, an ICS of IFN-γ was performed. For calibration purposes, an unstained control was analyzed. To control proper gating, unstimulated as well as GL9 peptide stimulated CTLs were detected.

D.5.1 The design of HIV-1 Gag derived antigens influences the capacity of the GL9 peptide to be cross presented

As detected for direct presentation, peptide cross presentation was not influenced by modest immunogen expression levels (Fig. D.16). Peptide presentation on mdDC occurred within a time period of 4 – 6 h post cocultivation initiation with infected HeLa cells. NYVAC-variants that initiated enhanced antigen expression in HeLa cells (see Fig. D.3 to D.5) did not induce cross presentation when being administered at MOI 5 (see Fig. D.16). Only NYVAC-gpn (ΔFS, Myr^-) and NYVAC-C, both encoding for the artificial GPN polyprotein, were shown to induce the GL9 CTL restimulation by cross presentation. Remarkably, NYVAC-C elicited a 4-fold higher CTL activation rate compared to NYVAC-gpn (ΔFS, Myr^-). Despite an increased Gag steady-state level in HeLa cells, introduction of a ribosomal frame shift strongly reduced the immunogen's cross presentation capacity (see Fig. D.4 and D.17 A). Due to their ability to form VLP, budding competent immunogens were designed to induce cross presentation. Surprisingly, peptides derived from budding competent antigens were not presented on mdDC (see Fig. D.17 B).

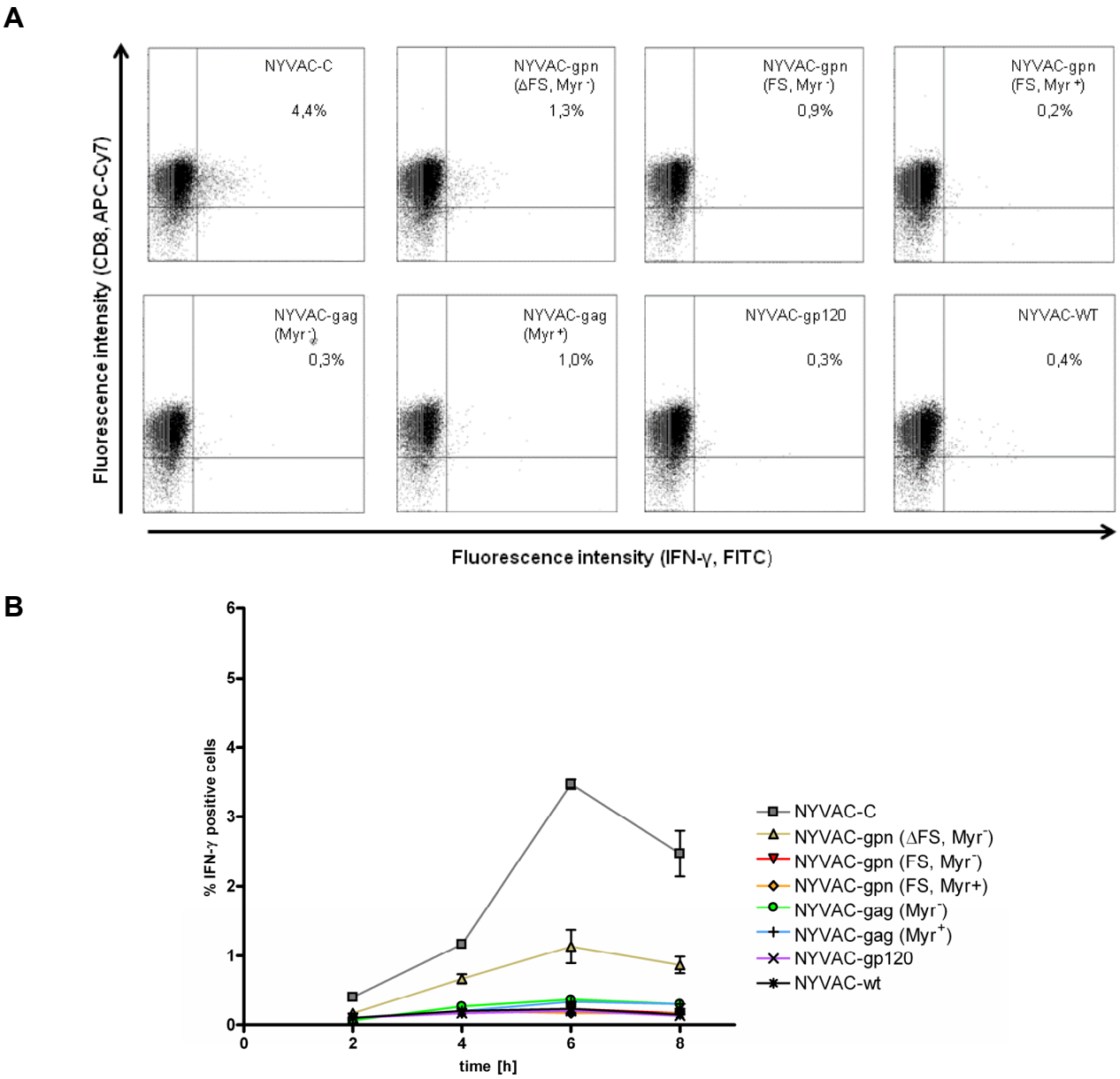


Fig. D.16 Vaccine candidate infected HeLa cells are capable of inducing GL9 cross presentation

$5x10^4$ HeLa cells were infected with the indicated vaccine candidates at MOI 5, incubated for 4 h and cocultivated with an equal number of mdDCs. Cocultivation with GL9 CTLs was initiated at the indicated time points. After a 10 h cocultivation period, $CD8^+$ CTLs were analyzed for IFN-γ expression by FACS analysis. *A*. Exemplary dot plot illustration of GL9 CTL restimulation by mdDC cocultivated for 6 h with vaccine candidate-infected HeLa cells *B*. Illustration $CD8^+$/IFN-$γ^+$ cells restimulation by mdDC cocultivated for the indicated time points with vaccine candidate-infected HeLa cells.

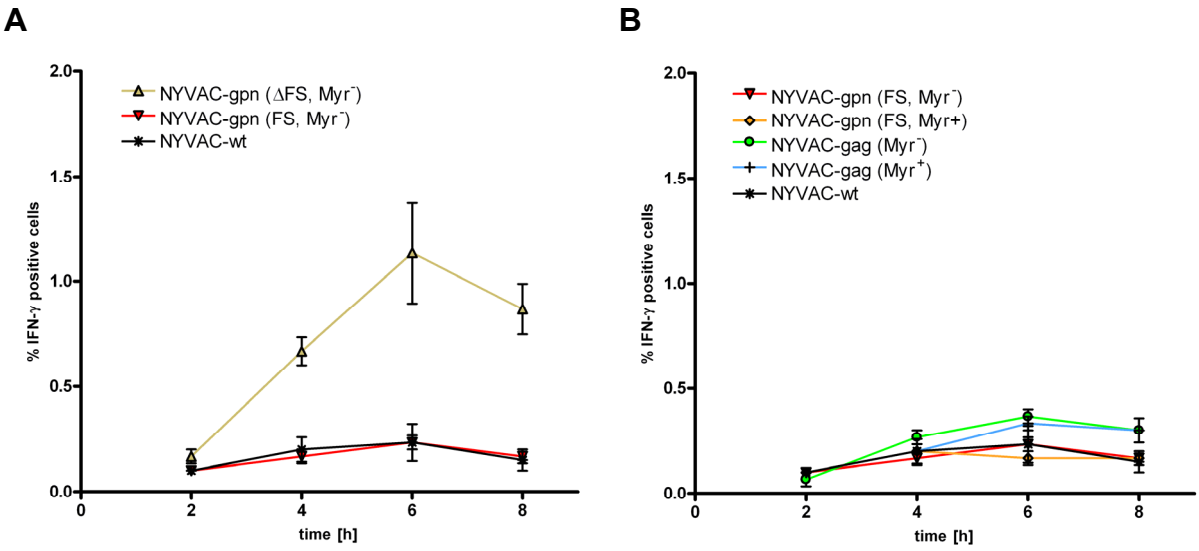


Fig. D.17: Cross presentation of GL9 is strongly influenced by immunogen composition and occurs within few hours post cocultivation

$5x10^4$ HeLa cells were infected with the indicated vaccine candidates at MOI 5, incubated for 4 h and cocultivated with an equal number of mdDCs. Cocultivation with GL9 CTLs was initiated at the indicated time points. After a 10 h incubation period, $CD8^+$ CTLs were analyzed for IFN-γ expression by FACS analysis. *A.* and *B.* Detailed illustrations of $CD8^+$/IFN-γ^+ GL9 CTL activation induced by selected vaccine candidate subsets.

D.5.2 The cross presentation efficiency of Gag derived peptides is strongly dependent on the MOI

In direct presentation assays, a strong dependency on virus multiplicity was observed (see D.4.2). Due to the marginal cross presentation capacity observed for some vaccine candidates, the effect of virus concentration on the magnitude of cross presentation was examined. When administering the vaccines at MOI > 10, an up to 10-fold increase in CTL restimulation was detected (see Fig. D.15). Similar to direct presentation, NYVAC-gpn (ΔFS, Myr^-) and NYVAC-C coding for GPN induced the most extensive CTL activation. The restricted cross presentation capacity of NYVAC-gag (Myr^-) could be circumvented by application of higher virus concentrations whereas the remaining antigens failed to induce considerable CTL restimulation even at highest MOIs tested.

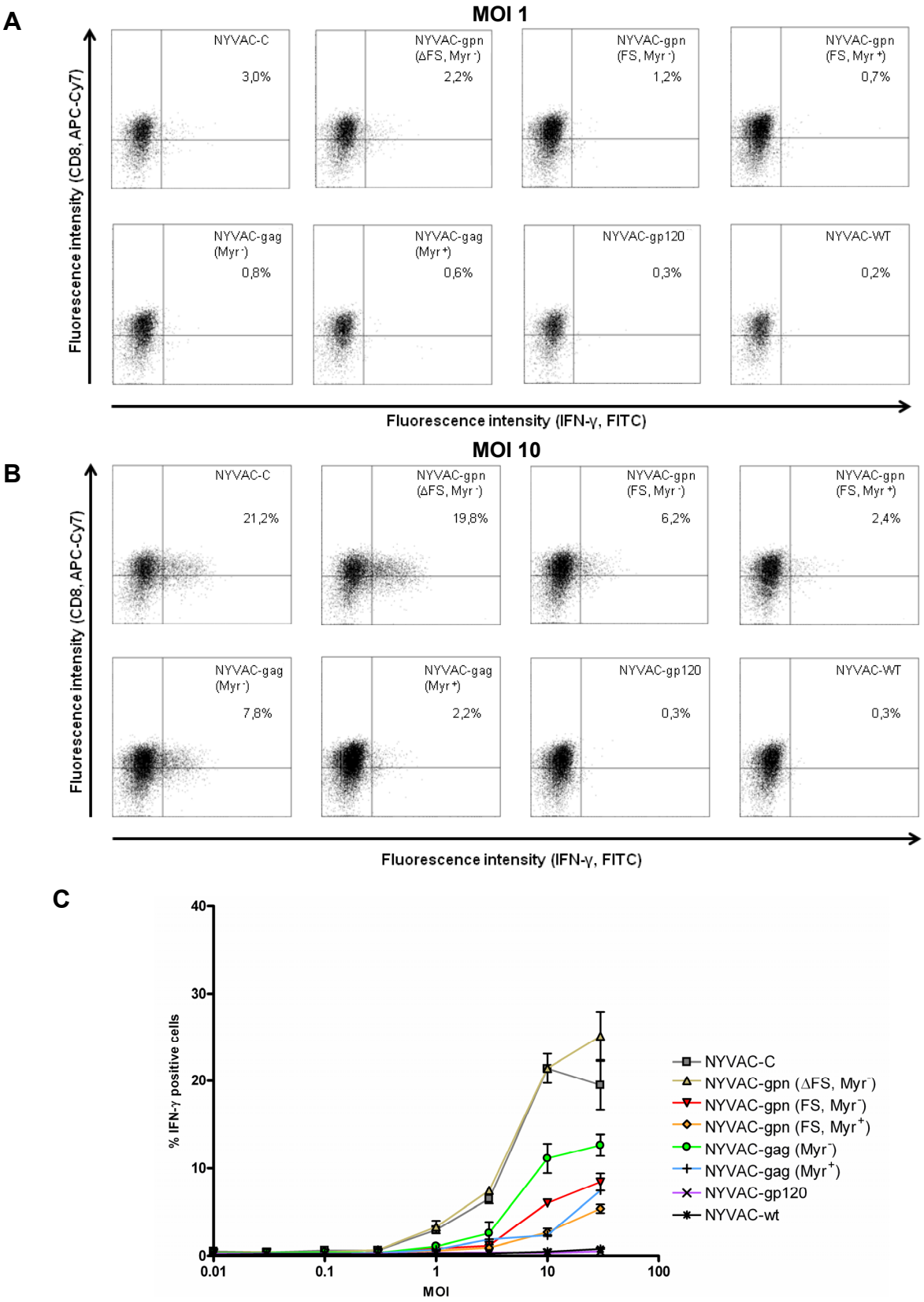


Fig. D.18: GL9 cross presentation requires vaccine candidate application in high concentrations

$5x10^4$ HeLa cells were infected with different vaccine candidates at the indicated MOIs, incubated for 4 h and cocultivated with mdDCs. Cocultivation with GL9 CTLs was initiated 4 h post HeLa / mdDC coculture initiation. After a 10 h incubation period, $CD8^+$ CTLs were analyzed to express IFN-γ by FACS analysis. *A.* and *B.* Exemplary dot plot illustration of GL9 CTL restimulation by mdDC infected by vaccine candidate infected HeLa cells. The cells were infected and incubated for 4 h each. T lymphocytes were analyzed to express both CD8 and IFN-γ. *C.* Illustration of $CD8^+$ GL9 CTL restimulation dependend on the virus multiplicity in a cross presentation assay.

D.5.3 The cross presentation of Gag derived peptides can effectively be driven by transfected HeLa cells and is not influenced the NYVAC vector

Based on the results obtained in the clinical phase-I studies EuroVacc01/02, the new generation antigens were designed in consideration of maximizing Gag accessibility. However, in direct and cross presentation assays the newly designed immunogens did not fulfil the desired characteristics. To determine whether the NYVAC-vector itself has an influence on cross presentation, an alternative immunogen delivery agent was tested. As a vector system, the DNA plasmid pcDNA3.1 was chosen. DNA vectors eliminate the peptide replacement induced by vector-own peptides. Since the monodirectional CMV promoter does not allow expression of two separate antigens, NYVAC-C encoding for gp120 and GPN can not be mimicked by pcDNA3.1. To allow comparison, infection and transfection rates of HeLa cells are required to be similar. Preliminary experiments demonstrated that a transfection rate of 90 % was achieved when transfecting $1x10^5$ HeLa cells with 1µg plasmid DNA. This corresponds to the infections levels obtained with NYVAC infection (see Fig. D2).

Consistent with the results shown above (see D.5.2), only the artificial polyprotein GPN was able to induce efficient cross presentation (see Fig. D.18). Cross presentation of GPN-derived GL9 peptides induced restimulation of approximately 6 % of the applied CTL. Modest GL9 presentation driven by pcDNA3.1-gpn (FS, Myr^-) transfected HeLa cells was observed. The remaining candidate vaccines did not induce CTL restimulation. Again, VLP formation capacity did not elicit cross presentation. Peptide presentation was observed to occur subsequently after plasmid DNA transfection. As shown for the NYVAC vector, GL9 presentation is declined 8 h post transfection. Thus, the obtained results mostly correspond to the conclusions drawn from the cross presentation assays driven by NYVAC infected HeLa cells.

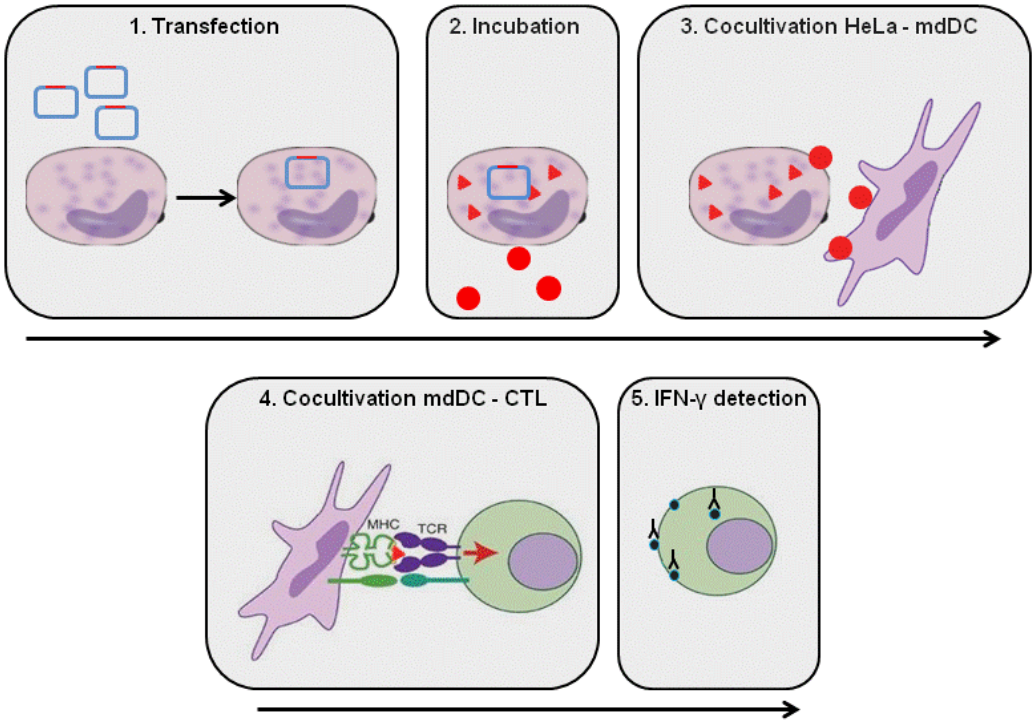
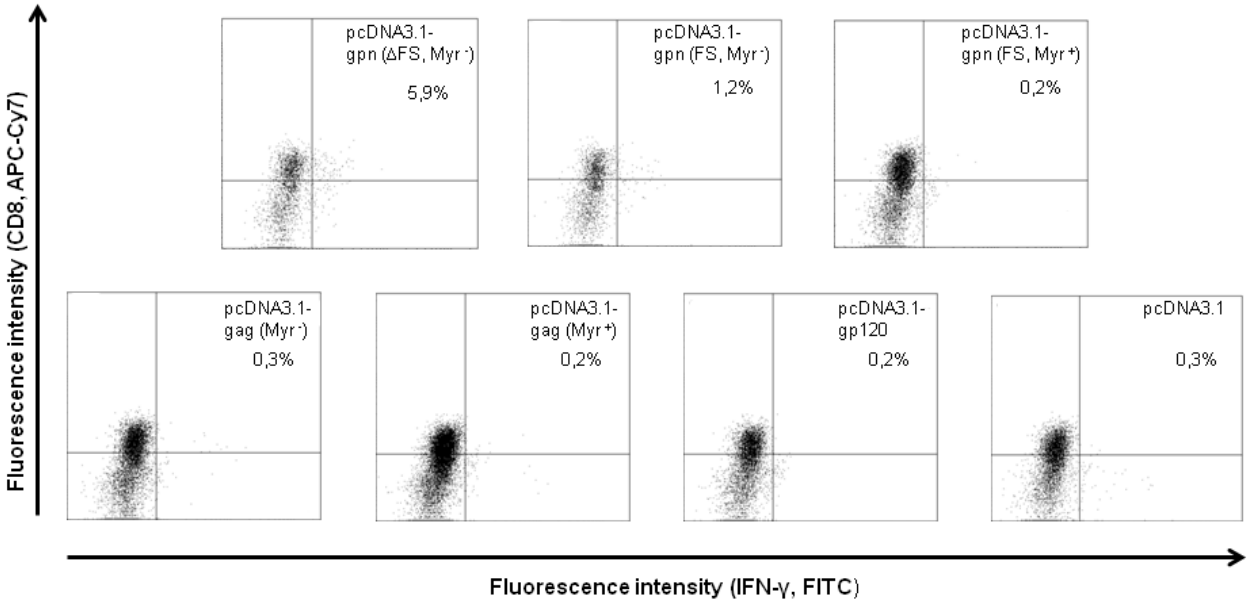


Fig. D.19 Experimental layout of cross presentation assays applying transfected HeLa cells

$1x10^5$ HeLa cells were transfected by 1 µg pcDNA3.1 coding for different HIV-1 derived antigens. The cells were incubated for 4 h. After incubation the HeLa cells were cocultivated with mdDC derived from a HLA-B*07$^+$ donor. After incubation for varying time points, the mdDC were cocultivated with an equal amount of a GL9-specific T cell clone. After an incubation period of 12 h, the T cells were evaluated to express IFN-γ by FACS analysis.

A



B

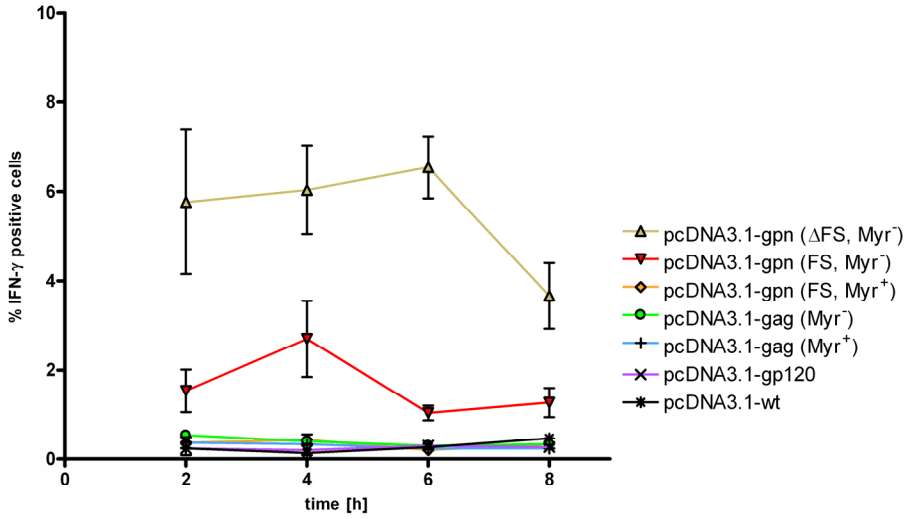


Fig. D.20 HeLa cells transfected with pcDNA3.1-gpn (ΔFS, Myr $^-$) induce most extensive GL9 cross presentation in mdDC

1*10^5 HeLa cells were transfected with 1 µg pcDNA3.1 coding for the indicated immunogens. The cells were incubated for 4 h and cocultivated with mdDCs for the indicated timepoints, subsequently followed by GL9 CTL cocultivation. The cells were incubated for 10 h. CD8$^+$ CTL were analyzed for IFN-γ expression by FACS analysis. *A*. Exemplary dot plot illustration of GL9 CTL restimulation driven by transfected HeLa cells. The cells were cocultivated with mdDC for a 4 h time period. After cocultivation, CTL were analyzed for IFN-γ expression. *B*. Illustration of CD8$^+$ GL9 CTL restimulation in a cross presentation assay dependent on the indicated HeLa – mdDC coculture incubation periods.

D.6 Cross presentation of the HIV-1 Gag-derived GL9 peptide is mainly driven by the uptake of cell-associated immunogens

For the induction of cross presentation, besides the protein half-life and amino acid sequence (see B.2.2.3), the accessibility of extracellular immunogens is described to be a limiting parameter [129]. The ability of Gag to form VLPs increases the amounts of extracellular immunogen that is available for cross presentation. VLPs consist of a lattice of immature Gag precursors surrounded by a host cell derived membrane and are able to self assemble into an organized particle structure. These particles represent a form of subunits vaccines that can efficiently induce cross priming [232,233,259]. Therefore, candidate vaccines able to induce VLP formation were developed. Surprisingly, in cross presentation assays these vaccine variants were shown not to elicit GL9 CTL restimulation. As described, phagocytosis of cell bodies derived from apoptotic cells was reported to trigger cross presentation [136,137]. Therefore, uptake and presentation of immunogens associated with cells or embedded in VLP was compared.

To analyze whether cross presentation is driven by either infected cells or VLP containing supernatants, both fractions were analyzed in a cross presentation assay separately (see Fig. D.21). HeLa cells were infected by the vaccine candidates at MOI 5. After virus removal 1 h post infection, cells were incubated for 3 h. The VLP containing supernatant was removed and both, HeLa cells and supernatant, were incubated on mdDC for 4 h. To detect cross presentation, the mdDC were co-cultivated with anti-GL9 CTL.

As described for influenza [104,138], DCs infected with infected HeLa cells were capable of stimulating specific CTL. As shown previously, only NYVAC-gpn (ΔFS, Myr^-) and NYVAC-C coding for GPN were able to induce cross presentation. For both vaccine candidates, CTL restimulation was mainly driven by the uptake of infected cells and cell debris. Nevertheless, the induced peptide presentation is reduced by a factor of 2.5 than compared with GL9 presentation induced by the uptake of cells together with the supernatant (see Fig. D.18). The remaining vaccine candidates did not restimulate considerable CTL amounts by cross presentation. Neither the uptake of infected HeLa cells nor extracellular immunogens induced cross presentation. Peptides derived from VLPs were not efficiently presented on mdDCs.

Since NYVAC was found to replicate in HeLa cells, to avoid direct presentation the cross presentation assay was developed not to exceed a duration of 8 h until CTL cocultivation was initiated. Thus, the immunogens present in HeLa cells or the supernatant cannot be quantified by Western-blot analysis or ELISA. Due to their insensitivity, immunogen expression can be detected not until an incubation period of at least 16 h.

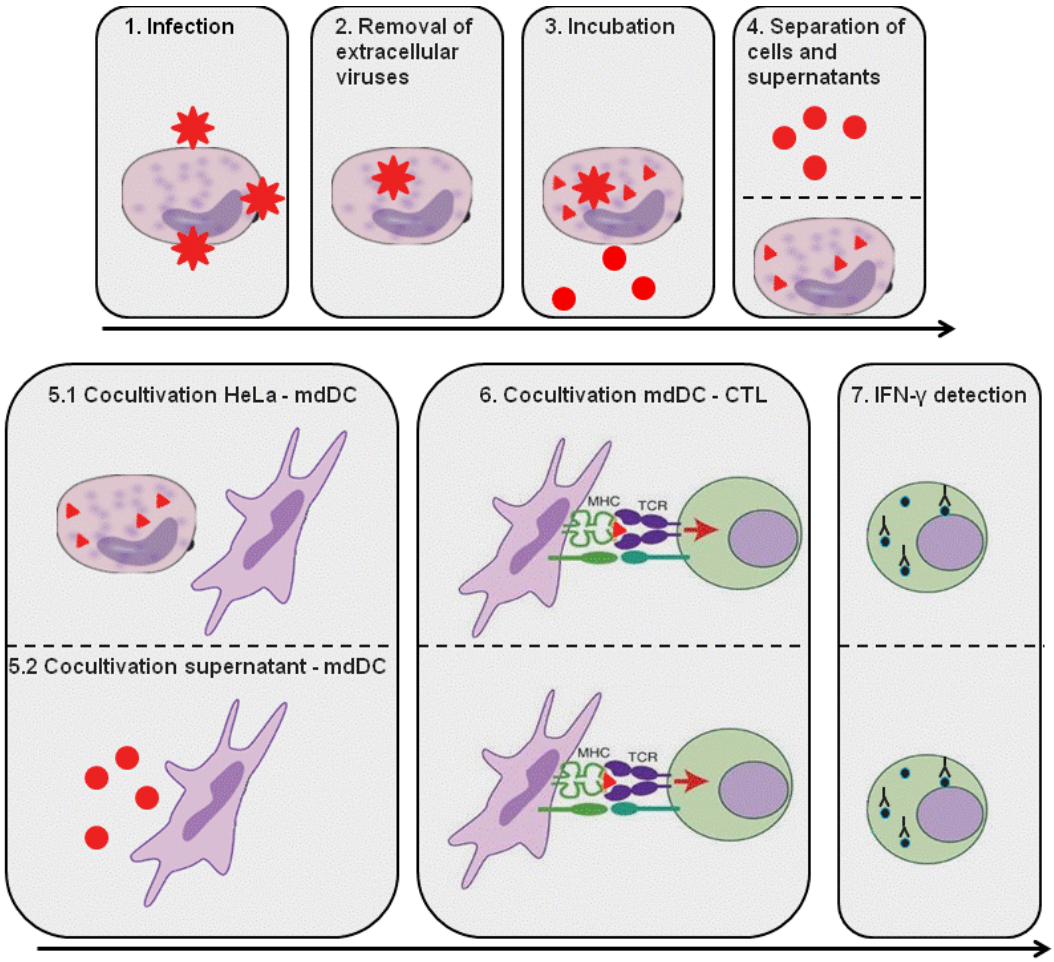


Fig. D.21 Experimental layout of cross presentation assays applying transfected HeLa cells

$1x10^4$ HeLa cells cells were infected with the vaccine candidates by MOI 5. After removal of extracellular viruses by five washing steps 1 hpi cells were incubated for 3 h. After incubation, the HeLa cells and supernatants were separated and cocultivated with mdDC derived from a HLA-B*07^+ donor. After incubation for 4 h, the mdDC were cocultivated with an equal amount of a GL9-specific T cell clone. After an incubation period of 12 h, the T cells were evaluated to express IFN-γ by FACS analysis.

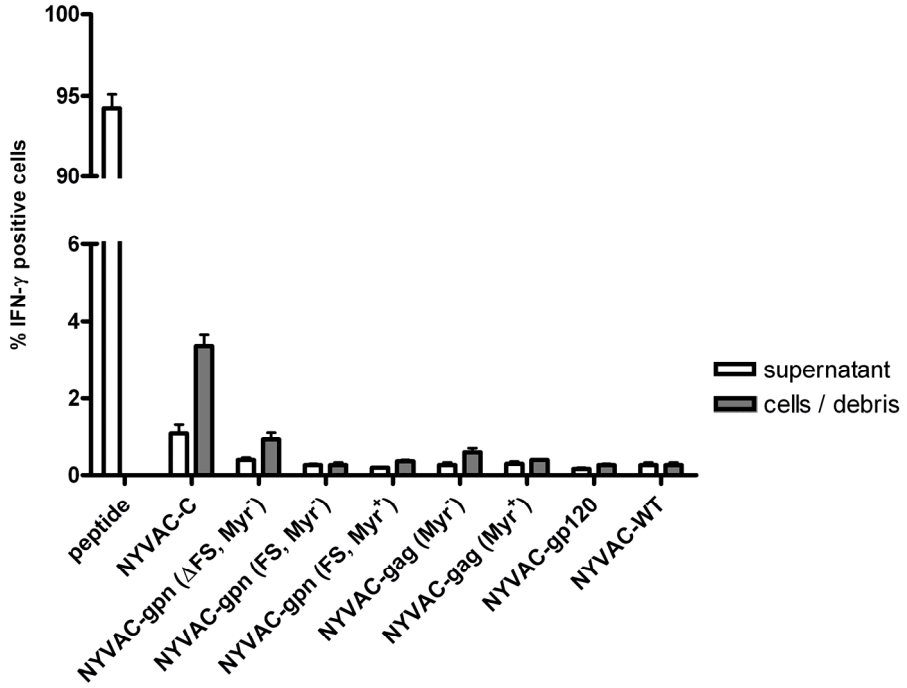


Fig. D.22 Cross presentation is mainly driven by the uptake of cell-associated immunogens by mdDCs

$5x10^4$ HeLa cells were infected with the indicated vaccine candidates at MOI 5 and incubated for 4 h. Cells and supernatant were divided and cocultivated with mdDC for 4 h, followed by GL9 CTL cocultivation. The cells were incubated for 10 h, $CD8^+$ CTL were analyzed for IFN-γ expression by FACS analysis.

D.7 Different peptides derived from the same HIV-1 antigen vary in their direct and cross presentation capacities

Antigen uptake, processing and presentation on professional APC are strongly dependent on the antigen composition (see B.2.2.2 and B.2.2.3). Since surrounding sequences and the peptide itself influence proteasomal digestion by positioning the proteasome's active site to distinct regions [151,154,155,260] not every possible peptide present in the antigen is generated by the processing machinery. Thus, it is likely that not every single peptide present in an immunogen is generated. Due to escape mutations, generation and presentation of several peptide could be inhibited. Therefore, direct and cross presentation of a second Gag-derived peptide was investigated. For characterization, presentation of a derivative of the best defined and examined HIV-1 epitope SLYNTVATL (SL9) was analyzed [228,229]. SL9 is an immunodominant HLA-A*2 restricted help-independent epitope derived from Gag-p17 (residues 77-85). SL9 is found in approximately 70% of all HLA-A*2 patients in acute HIV infection whereas in chronic infection only a small fraction of patients presented this epitope [230]. Due to variations in the amino acid sequences in the 97CN54 and 97CN001 isolates, a T cell clone recognizing the HLA-A*2

restricted peptide SL**F**NTVAT**P** was utilized. The direct and cross presentation assay was performed as described previously (see Fig. D.14 and D.19).

Surprisingly, neither direct nor cross presentation of the SP9 peptide could be induced by the different immunogens (see Fig. D.23). Although stimulation with the corresponding peptide induced restimulation of the SP9 CTLs, no specific immunogen-driven restimulation was observed. Since none of the vaccine candidates induced peptide presentation, it is likely that peptide generation or presentation is suppressed due to immune escape mutations. Certainly, compared to the GL9 CTLs, unspecific SP9 CTL restimulation was determined to be quite pronounced. For discussion, see E.3.4.

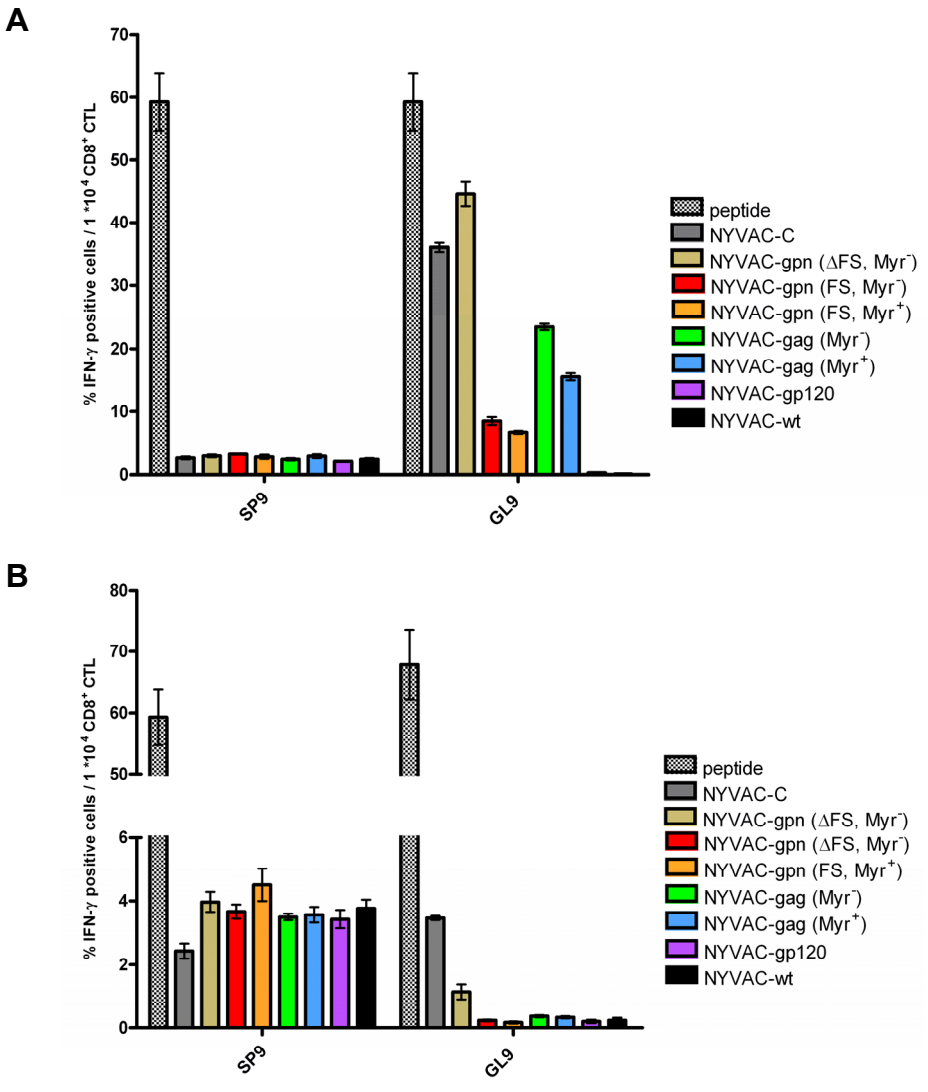


Fig. D.23 The HIV-1 p17 peptide SP9 is not presented on mdDCs by direct and cross presentation

A, SP9 direct presentation: $5x10^4$ mdDCs were infected with the indicated vaccine candidates at MOI 5, incubated for 6 h and cocultivated with an equal number of SP9 / GL9 specific CTL for 10 h. $CD8^+$ CTLs were analyzed for IFN-γ expression by FACS analysis.

B, SP9 cross presentation: $5x10^4$ HeLa cells were infected with the indicated vaccine candidates at MOI 5 and incubated for 4 h. The HeLa cells were cocultivated for 4 h with $5x10^4$ mdDC followed by cocultivation with $5x10^4$ SP9 / GL9 CTL. The cells were incubated for 10 h, $CD8^+$ CTLs were analyzed for IFN-γ expression by FACS analysis.

E Discussion

E.1 The vaccine candidates allow expression of HIV-1 derived immunogens in HeLa cells and human mdDCs

So far, no effective HIV-1 vaccine based on the generation of neutralizing antibodies could be developed. Due to its partially protective properties, Gag-specific T cell responses are considered to be a promising approach for induction of immunity against an HIV-1 infection (see B.1.3.2. and B.2.3.2). The vaccine candidate NYVAC-C was designed as a multiepitopic bivalent vaccine consisting of both Gag/Pol/Nef (GPN) and gp120 based on the C-clade 97CN54 provirus isolate encoded in a poxviral NYVAC vector [243]. When analyzing the immunogenicity of the HIV-1 antigens GPN and gp120 in the phase-I clinical trials EuroVacc01 and EuroVacc02, the induced T cell responses were mainly directed against Env. Gag- and Pol-specific T cells were only detected at low levels. Thus, a new generation of immunogens based on 97CN54 GPN and Gag was developed that were designed to elicit increased Gag expression and therefore enhanced immunity. Using NYVAC-C as a reference construct, several immunogen modifications such as (i) size reduction, (ii) modulation of Gag versus GPN ratios and (iii) VLP formation capability were introduced. The vaccine candidates were designed to elicit efficient immunogen expression in tissue cells and professional APC to induce improved direct and cross presentation. In this thesis the influence of the modifications on antigen expression as well as direct and cross presentation was analysed and compared to NYVAC-C.

First, due to extensive immunodominance of the envelope protein, gp120 was deleted from the vector. As predicted, gp120 elimination resulted in increased amounts of intracellular p24. Notably, due to Env driven apoptosis [257] (see Fig. D.6), in NYVAC-C infected mdDCs, Gag production was completely abolished 8 hpi. The results indicate that apoptotic cells fully disable NYVAC replication. In HeLa cells, deletion of gp120 enhances the GPN expression (see Fig. D.3). Nevertheless, by NYVAC-C replication detected in HeLa cells it can be demonstrated that Env mediated apoptosis is strongly dependent on the cell type [261].

For enhanced Gag expression, the naturally occurring -1 ribosomal frame shift was reconstituted in the artificial GPN polyprotein. In HeLa cells, insertion of a ribosomal frame shift into GPN induced an at least 10-fold increase of intracellular Gag steady-state levels (see Fig. D.3). The deletion of PN resulted in reduced antigen size and reconstitution of the natural Gag protein-shape. The size-reduced immunogens were expressed more extensively.

Due to its large complexicity and artificial origin, (i) expression, (ii) folding and (iii) proteasomal degradation of GPN may be altered compared to Gag. Large proteins are frequently described to be demanding in production [262]. Additionally, GPN folding might be unfavourable for the

immunogen expressing cells. This could lead to rapid protein depletion by proteasomal digestion that occurs via the ubiquitination pathway (see B.2.2.3.1). Alterations in protein folding can redirect ubiquitination complexes resulting in more rapid cleavage [117,147]. Rapid co-translational digestion initiated by misfolded proteins reduces the intracellular immunogen amount [263].
For further size-reduction, vaccine candidates coding for Gag were developed. When analyzing antigen expression, the NYVAC-gpn (FS, Myr^-) induced translation of Gag and GPN in a 95:5 ratio was strongly diminished compared to production of Gag (Myr^-) in HeLa cells (see Fig. D.3). This indicates, that expression of GPN limits overall antigen expression. Based on the observation that even small GPN levels induced a potent decline of detectable p24, a cytotoxic effect of GPN can be assumed. As described before, due to limited antigen expression in NYVAC infected mdDCs, this influence can basically be detected in vaccine treated HeLa cells resulting in an almost 20-fold increased intracellular p24 concentration (see Fig. D3 A).
To facilitate cross priming of antigen-derived peptides, vaccine variants coding for budding competent Gag were designed. In cells infected by NYVAC-gpn (FS, Myr^+) and NYVAC-gag (Myr^+), VLP formation significantly diminished the intracellular p24 concentration. Subsequently after translation, Gag is transported to the cell membrane mediated by cellular ESCRT complexes [264,265]. There, the myristoylated proteins are anchored into lipid raft regions in the plasma membrane which, together with oligomerization promotes, virus particle assembly [21,22]. Thus, the 5-fold reduced p24 concentration induced by budding competent antigens can be attributed to rapid Gag export (see Fig. D.5). This suggests that antigen cross presentation capacity is enhanced on the cost of direct presentation ability (see E.3).

Transgene expression was observed to occur in a short time-frame early after infection in both mdDCs and HeLa cells. Despite the early expression, minimal immunogen amounts were detected in mdDCs. In NYVAC-infected DCs, protein synthesis of late gene products is blocked [79,80,81]. Since immunogen expression is controlled by an early/late (E/L) promoter, their transcription is aborted when reaching late infection phases resulting in declined production. Thus, abortive transcription masks differences in expression driven by the immunogen's characteristics. Low-level protein synthesis and the transcriptional block of late genes do not allow extensive expression of immunogens. Therefore, the influence of frame shift insertion in the GPN polyprotein could not be observed in NYVAC infected mdDC (see Fig. D.7 A).

Taken together, size-reduced immunogen variants triggered the best antigen expression in APC. Thus, most efficient peptide direct presentation can be suggested for these immunogens. Due to reduced steady-state levels of intracellular Gag proteins elicited by budding competent antigens, VLP formation could result in efficient peptide cross presentation.

E.2 NYVAC-induced partial mdDC maturation is transgene independent

The major goal in HIV-1 vaccine research is to develop a vaccine that induces long-lasting mucosal and systemic immunity. Since viral proteins are often poorly immunogenic, various adjuvants have been developed to efficiently enhance immune responses [266,267]. By administration of subunit vaccines embedding HIV-derived transgenes in an immunogenic viral vector backbone efficient immune responses can be generated without applying additional adjuvants [268].

For efficient T cell activation, upregulation of peptide presenting MHC molecules as well as co-stimulatory molecules such as CD80 and CD86 (see B.2.2.1) is required. Due to the expression of viral immune regulators, NYVAC infected mdDCs do not undergo a complete maturation process. These regulators mainly target cytokines, chemokines and TLR-signalling pathways [84]. To analyze whether the immunogenic properties of the new generation antigens support NYVAC induced mdDC maturation, upregulation of different maturation markers induced by the vaccine candidates was monitored.

The co-stimulatory molecules CD80 and CD86 (also known as B7-1 and B7-2) were not upregulated during the maturation process (see Fig. D.8 A to D). However, upregulation of the antigen-presenting molecule HLA-DR was observed 12 - 24 h post NYVAC infection. Since NYVAC-induced HLA-DR surface concentration did not differ from that elicited by the strong stimulant LPS the activation can be considered not to be increasable anymore. At later stages of infection, a decline of surface maturation markers was monitored. Due to the observation that LPS stimulated mdDC did not downregulate surface presentation of CD80 / CD86 and HLA-DR, a NYVAC driven immune escape mechanism can be concluded. Since efficient T cell priming requires extensive DC maturation, the downregulation of CD80 and CD86 48 hpi indicates that T cell priming is limited to occur within 24 hpi.

When examining the vaccine candidates' influence on mdDC maturation, no significant differences between the vaccines were detected 24 h post DC infection (see Fig. D.8). A decreased surface presentation of maturation markers 48 hpi was found for all candidate vaccines except NYVAC-gpn (ΔFS, Myr$^-$). Nevertheless, these data suggest that the antigen's influence on maturation is negligible. Therefore, different CTL restimulation capacities are not dependent on mdDC maturation states induced by the vaccine candidates.

In summary, NYVAC induced maturation was shown to remain incompletely. Upregulation of the co-stimulatory molecules CD80 and CD86 was not observed. Due to immune escape mechanisms, surface presentation of maturation markers was strongly reduced 48 hpi. The antigen itself did not influence maturation or at least only marginally. Since poxviral vectors were used for vaccination efficiently (see B.3), T cell priming is not inhibited by incomplete maturation. Thus, the vaccine candidates induced CTL restimulation was analyzed further.

Admittedly, immune tolerance against HIV derived antigens induced by immature DCs might elicit undesired side effects. As described by Mahnke *et al*., peptide presentation of immature DCs is involved in the maintenance of peripheral tolerance, including the induction of T cells with regulatory properties [269].

E.3 GL9 derived from the artificial GPN polyprotein is presented most efficiently

Generation of effective CTL responses, a vaccine has to induce antigens presentation efficiently. For direct and cross presentation, immunogens present in the cytoplasm are processed proteasomaly into peptides. The peptides are transported into the endoplasmatic reticulum where they form pMHC complexes that are presented at the surface of DC. These complexes can specifically be recognized by T cell receptors (TCR) of CTLs. pMHC recognition by specific TCR induces CTL activation [119,120]. Besides control of the cell cycle and metabolic pathways [118], proteasomal protein processing in particular plays a role in degrading misfolded, unfolded, damaged or mutated proteins [116,117].

Peptide formation by protein proteolysis is directly influenced by the protein's (i) amino acid sequence and (ii) pro-inflammatory environment. Amino acid variations alter ubiquitination and proteasomal digestion patterns [144,145,146]. Thus, small differences in immunogen composition can result in altered peptide processing and cleavage rates [117]. Antigen-induced cytokine expression can alter proteasome composition and therefore influence degradation [151,156,270]. Notably, variations in proteasomal processing can be influenced by peptide flanking regions [154,155]. An immunogen's cross presentation capacity is strongly influenced by structure and amino acid sequence and length of the peptide's precursor protein [139,140].

E.3.1 The NYVAC vector induces rapid peptide presentation but causes undesired effects when being administered at high concentrations

E.3.1.1 Antigen presentation occurs rapidly and is mediated by incompletely maturated DC

In both, the direct and cross presentation assay, GL9-specific CTL restimulation was observed. Although poxviral vectors were described to mainly stimulate T lymphocytes by cross priming [142], direct presentation of the GL9 peptide was shown to occur indeed. With NYVAC-infected mdDC, up to 30% of the administered GL9 CTL could be activated. Since CTL stimulation requires a minimum of several hundreds of pMHC for activation [271,272], efficient GL9 presentation on the APC can be considered. With regard to the large amount of (i) alternative HIV-1, (ii) NYVAC derived

peptides, and (iii) self-peptides presented on vaccine candiate infected DC, direct presentation can be estimated to occur dominantly.

Remarkably, presentation of GL9 peptides occurs early after mdDC infection with the vaccine candidates or infected HeLa cells. As described [272,273], peptide presentation was observed already 4 h post infection in low and high MOI infections,. Therefore, since mdDCs are found to maturate completely within 24 hpi, CTL stimulation by either immature (iDC) or not completely maturated DC can be hypothesized [83]. On one hand CTL restimulation by iDC could be a benefit as it allows T cell activation induced by low peptide presentation levels. On the other hand, low-level antigen presentation by iDC is involved in the generation of immune tolerance. Thus, iDC can induce T cells with regulatory properties that are suppressing immune responses and therefore suppress protectivity [269].

Aside from the hypothesis, that iDC induce GL9 CTL restimulation, it can be suggested that the co-stimulatory molecules CD80 and CD86 are not required for T cell activation *ex vivo*. Since upregulation of HLA-DR but not CD80 and CD86 was observed, the necessity of the co-stimulatory molecules to induce GL9 specific CTL is doubtful. Pardigon *et al*. observed that naive $CD8^+$ T cells are fully activated by pMHC complexes alone, but that co-stimulation lowers their activation threshold [274]. Referring to these findings, a boosted maturation could induce more potent T cell responses.

E.3.1.2 For induction of direct presentation but avoiding apoptosis, a balanced administration of NYVAC-based vaccines is required

For both direct and cross presentation, a strong influence of the NYVAC concentration on GL9 CTL restimulation was detected. In direct presentation assays, no decline over time could be determined in GL9 presentation when applying low MOI (see Fig. D.11 B and D.13). However, when administering high virus multiplicities diminished peptide presentation is detected 8 hpi (see Fig. D.10 C and D.13). The decline of GL9 presentation could be attributed to peptide dissociation as well as a limited peptide half-life of commonly about 6 h [275,276]. Due to apoptosis induced by high virus multiplicities, the supply of newly generated peptides is interrupted. Together, modest protein half-life and NYVAC cytotoxicty decrease antigen presentation strongly. However, since APC migration from tissues to lymph nodes is described to occur within one hour [277], the small time span of peptide presentation is most likely sufficient for CTL stimulation.

Due to the observed cytotoxic effects, enhancing direct presentation by administering high virus concentrations appears to be inapplicable. When applying NYVAC in a concentration exceeding MOI 5, a strong decrease of antigen presentation was observed (see Fig. D.13). The synchronous decline observable for all vaccine candidates indicates that cytotoxicity mediated by the vector backbone but not by the antigen triggered apoptosis (see Fig. D13). However, due to extended apoptosis rates elicited by high MOI infections, the optimal balance between induction of strong

immune responses and avoiding cytotoxic effects has to be found for direct presentation. When administering NYVAC-based vaccines by injections, local virus concentrations will likely exceed multiplicities found to be toxic *in vitro*.

In cross presentation assays, high NYVAC multiplicities do not induce a decline in GL9 presentation. In fact, higher virus concentrations were shown to increase peptide cross presentation strongly. Efficient cross presentation is believed to take place in the lymph nodes only [278]. Cross presentation of pathogen-derived peptides by cells neighbouring infected cells in tissues would expose these cells to CTLs. To prevent uninfected but cross presenting cells in tissues from CTL induced apoptosis, tolerance against low-level presentation is strongly required. Thus, the observed requirement for high immunogen doses in cross presentation could represent a protective mechanism.

In summary, the virus multiplicity can direct the character of peptide presentation. When administering low virus amounts, direct presentation is favoured whereas high virus concentrations induce mainly cross presentation. Thus, a two-area peptide presentation model can be suggested when applying NYVAC-based vaccines by injection. At the entry sites, due to high virus amounts mainly cross presentation might be favoured while at distant regions, mostly direct presentation occurs.

E.3.2 The new generation antigens do not exhibit enhanced immunogenic properties

E.3.2.1 Enhanced proteolysis induced by non-physiological folding results in enhanced GPN immunogenicity

When comparing the vaccine candidates concerning their ability to induce antigen presentation, NYVAC-gpn (ΔFS, Myr^-) and NYVAC-C coding for GPN together with gp120 are found to induce the most potent direct and cross presentation. Although both variants were shown to elicit only modest antigen expression in mdDC as well as HeLa cells, they were able to activate GL9 specific CTLs extensively. Since mainly the weakly expressed artificial GPN polyprotein gave rise to GL9 presentation, efficient antigen presentation did not depend on antigen expression. Rather than the amplitude of antigen expression, antigen stability was described to be determining efficient peptide presentation [279,280]. Protein degradation is believed to be the rate-limiting step for adequate antigen presentation by MHC class-I molecules [281,282]. For instance, a metabolically unstable HIV-1 Nef protein was shown to induce enhanced CTL stimulation accompanied by efficient antigen presentation [283].

Since GPN is simply designed as a large polyprotein neglecting proper folding of the corresponding subunits, translation is likely to result in a protein with non-physiological shape.

(i) Protein fusions without employing spacer peptides, (ii) scrambling of Nef C- and N-termini, (iii) interruption of the RT reading frame, and (iv) insertion of amino acid substitutions probably lead to severe protein misfolding. Because of exposure of hydrophobic amino acid residues usually embedded in inner regions, misfolded proteins are often insoluble and therefore aggregate in the cytoplasm. *In vivo*, these protein accumulations can result in diseases that range from cancer and diabetes to neurodegeneration [284,285]. Thus, protein quality control functions to ensure that damaged and misfolded proteins are maintained at low amounts to limit their cytotoxic effect [286]. These defective ribosomal products (DRiPs) represent a large fraction of newly synthesized peptides [287]. Hence, efficient protein monitoring that comprises refolding by chaperones and proteasomal degradation is required to sustain normal cell functions [288].

Due to its extensive intramolecular modifications, chaperone-mediated protein quality control can presumably not reconstitute a native protein shape of the GPN polyprotein. Therefore, other than the notably stable Gag protein that displays a metabolic half-life of approximately 6 h [289], GPN is likely to be digested rapidly. As described, proteolytic antigen processing is often performed co-translationally or subsequently after translation [263,290]. Proteostasis of misfolded proteins is selectively driven by ubiquitination which guides proteins to cytosolic 28S proteasomes for degradation [291,292]. Thus, rapid proteasomal digestion and not poor expression levels could evoke the low GPN amounts detected in Western blot and FACS analysis (see Fig. D.1 and Fig. D.3). Next to non-physiological GPN shape, the target protein's amino acid sequence (e.g. degron signals) strongly influences the modification process [144,145,146] resulting in increased proteasomeal digestion rates. Hence, enhanced accessibility of immunogen derived peptides and therefore broad antigen presentation is elicited [117]. Thus, by modified amino acid sequences, GPN could be ubquitinated, processed and therefore presented more efficiently than the correctly folded Gag.

Besides altered proteasomal cleavage rates, variations in the amino acid sequence of Gag and GPN could induce differential proteasomal cleavage patterns. When entering the proteasome, the internal residues P1, P4 and P5 adjust the peptide in the active site of the proteasome which determines processing [151]. Since the proteasome is described to be a "multicatalytic protease" [148,149 ,150], variations in complex positioning on the target elicit different protein digestion results. Additionally, degron- or PEST-sequences inserted in the GPN polyprotein by chance could enhance degradation [153]. Based on cleavage variations evoked by the amino acid sequence, peptide formation is likely to be altered. Alternative peptide composition can affect downstream mechanisms involved in peptide presentation. Tap-mediated peptide translocation from the cytoplasm to the ER [160] as well as formation of pMHC complexes are strongly influenced by the transgene itself [165,166,170] (see B.2.1.3.).

Taken together, overall folding variations between Gag present in its native conformation and GPN captured in a non-physiological protein shape can be considered to be accountable for extensive differences in direct presentation. It is likely that protein-folding differences induce

variations in (i) patterns and rates of proteasomal digestion, (ii) TAP transport, and (iii) pMHC complex formation resulting in different or reduced peptide presentation. Thus, as described by Wong *et al*., the poor immunogenicity of wildtype Gag can be circumvented by targeting defective or unstable Gag variants to the proteasomal pathway for rapid degradation [293]. It has already been shown, that Gag variants enriched for degron signals induced an enhanced T cell stimulation [289]

E.3.2.2 Low GPN expression induced by insertion of a ribosomal frame shift strongly reduces its immunogenicity

For MHC class-I presentation, protein degradation is described to be the rate-limiting step for efficient antigen presentation [281,282] whereas the amount of antigen expression is reported to have minor influences [279,280]. However, when comparing NYVAC-gpn (ΔFS, Myr^-) and NYVAC-gpn (FS, Myr^-) a strong decrease in direct and cross presentation was observed for GPN (FS, Myr^-). Since reconstitution of the ribosomal frame shift strongly diminishes GPN expression [250], a correlation between antigen expression and peptide presentation is likely. Thus, to enhance an antigen's immunogenicity it has to be optimized for both expression and proteolysis. However, due to its size of 160 kDa and extended non-physiological structure, cytopathic effects induced by GPN might appear when present at too high concentrations.

E.3.2.3 Gp120 elicits immune stimulation in mdDC resulting in enhanced antigen cross presentation

It has been controversial whether the application of gp120 in a vaccine designed to elicit CTL responses is reasonable. Due to enlarged number of possible CTL epitopes, broader immune responses could be induced. Certainly, co-administration of HIV-1 gp120 was found to strongly diminish antigen expression in APC (see D.3.1 and D.3.2) as well as Gag CTL responses in both humans and mice [85,294]. Therefore, gp120 is believed to suppress efficient generation of CTL responses directed against HIV-1 Gag.

Surprisingly, despite abortive antigen expression NYVAC-C infected mdDCs are able to stimulate CTLs extensively by direct presentation (see Fig. D.11 and Fig. D.13). Thus, cytotoxic properties of gp120 observed in NYVAC-C infected mdDC (see E.1 and Fig. D.6) do not inhibit direct presentation. As described, the amplitude of antigen expression is shown once more not to be the major rate-limiting step for efficient antigen presentation by MHC class-I molecules. However, when comparing NYVAC-C and NYVAC-gpn (ΔFS, Myr^-) no gp120-mediated benefit on direct presentation can be detected (see D.11 *A* and *B*). Similarly, gp120 does not influence cross presentation in early stages (up to 4 h) of infection (see D.16 B). NYVAC-C only induced

enhanced cross presentation observed for extended HeLa-mdDC cocultivation periods. In fact, a prolonged Env incubation time on mdDC seems to increase vaccine immunogenicity.

HIV-1 Env was described to interact with pDCs and mdDCs via the surface receptor DC-SIGN. Once taken up, gp120 can influence immune stimulatory signalling cascades by interaction with various Toll-like-Receptors (TLR) [295]. Furthermore, gp120 was shown to affect mdDC maturation and cytokine expression [296,297]. Cytokines have immune-stimulatory properties and can assist in T cell stimulation. An altered cytokine environment can enhance the vaccine's immunogenicity. Additionally, gp120 was described to induce IFN-γ expression in human PBMC [296]. IFN-γ influences the proteasome composition resulting in changed catalytic properties [157]. These immunoproteasomes possess altered substrate properties as well as an enhanced cleavage rate [156]. Hence, Env driven IFN-γ expression could result in enhanced or altered peptide presentation and therefore CTL restimulation. Notably, gp120 induced alterations in TLR signalling were observed to mainly influence cross presentation [298,299] whereas induction of immunoproteasomes is not exclusive for cross presentation. Thus, the observed gp120 benefit on cross presentation is likely not driven by IFN-γ induced immunoproteasomes.

Taken together, HIV-1 Env influences a vaccine's immunogenicity. As shown, peptide presentation can be modulated by gp120 application. Thus, application of gp120 as an immunogen has to be adapted to the applied vector system. When using vectors that induce immuno-responses by cross presentation, the co-application of gp120 could boost CTL stimulation.

E.3.2.4 VLP formation does not benefit cross presentation

VLPs represent a form of subunit vaccines that are able to self assemble into an organized particle structure [232,233]. Since they can efficiently induce cross presentation of HI-viral antigens [234,235] several new generation antigens were designed to induce VLP formation.

Compared to budding incompetent antigens, reduced peptide presentation was observed for immuogens that allow VLP formation. Since the p24 steady-state level in mdDC was shown to be diminished by VLP budding (see Fig. D.7), it can be concluded that the Gag budding process influences direct presentation. Since the physiological shape of Gag was conserved when the vaccine candidate's antigens were designed (see Tab. D.1), co-translational proteolysis can be considered to occur rarely. When comparing the protein's metabolic half-life of 6 h [289] with the short time span of 5-10 min required for Gag budding [300], it can be concluded that VLP formation takes place before protein degradation occurs. Thus, assembly of Gag particles in the cytoplasm as reported for non-myristoylated G2A variants [301] enhances accessibility for proteasomal degradation and therefore direct presentation.

The reduction in direct antigen presentation upon VLP formation was taken due to enhanced cross presentation. Certainly, VLPs derived from NYVAC infected HeLa cells were shown not to

infect mdDC efficiently. As described, cross presentation is mainly driven by the uptake of apoptotic bodies or infected cells [104,138]. Antigen presentation induced by HIV-1 Gag derived VLP was rarely described to stimulate CTLs [302]. In fact, VLPs were observed to elicit mainly $CD4^+$ T helper cell and antibody responses, sometimes misleadingly entitled as cross presentation [303]. Admittedly, induction of VLP-driven immune responses usually requires additional adjuvants [304]. Since VLPs are often obtained from baculoviral expression systems, immune stimulation observed in mice might be induced by non-autologous proteins [305]. Thus, co-administration of adjuvants could induce cross presentation elicited from VLP produced by autologous cells. Nevertheless, the poor immunogenicity of Gag (see E.3.1.3) can not be circumvented by this. Rapid, cotranslational proteolysis, required for optimal antigen presentation (see E.3.1.3), is likely to inhibit VLP formation.

Taken together, GL9 derived from the artificial polyprotein GPN was observed to be presented most efficiently on APC by direct and cross presentation. It can be supposed, that due to misfolding GPN is processed more efficiently than the correctly folded new generation immunogens.

E.3.3 Antigen immunogenicity is not vector dependent

The new generation vaccines were designed to elicit higher Gag expression since it was estimated that higher Gag amounts would allow for elevated direct and cross presentation. Surprisingly, the new generation immunogens did not induce efficient antigen presentation on mdDC. Because NYVAC is described to possess multiple immune-escape mechanisms [79,83], its influence on antigen presentation was analyzed. Moreover, for the NYVAC related poxviral MVA vector, immunodominant vaccinia-specific CTL responses were shown to limit the effectiveness of poxviruses in recombinant vaccination strategies [306]. Due to immune-escape mechanisms, even dominant peptides are frequently not capable of mediating protection against infections. Furthermore, the presentation of these unprotective immunodominant peptides can inhibit the presentation of protective subdominant epitopes [222].

To determine whether NYVAC influences or varies cross presentation, an alternative vector system was analyzed. To limit immune-escape and application of non-HIV-1 derived antigens, plasmid DNA was chosen as a delivery system for the newly designed immunogens. Since transfection of mdDC is ineffective and might influence maturation, GL9-presentation was only observed in cross presentation assays. Again, only the artificial polyprotein GPN induced efficient cross presentation (see Fig. D.20). However, pcDNA3.1-gpn (FS, Myr^-) transfected HeLa cells were able to modestly induce GL9 cross presentation. Thus, no extensive variations in cross presentation induced by NYVAC infected or plasmid DNA infected HeLa cells were detected.

Therefore, it can be concluded that NYVAC driven immune-escape does not affect antigen presentation. Secondary, since immunogen application by plasmids does not enhance antigen presentation a replacement of the GL9 peptide by NYVAC derived epitopes can be excluded.
In summary, the data indicate that NYVAC does not influence an antigen's direct and cross presentation capacity, although NYVAC possesses broad immunogenic potential and strongly influences mdDC maturation.

E.4 Not every single peptide theoretically covered in an immunogen is efficiently presented on APC

For induction of broad immune responses and circumvention of escape mutations, epitope enrichment within a single immunogen is discussed to be favourable. So-called mosaic antigens are designed to optimize cellular immunologic coverage of global HIV-1 sequence diversity [220]. These immunogens are assembled from fragments of natural sequences derived from viruses of different clades [220]. For the development of an all-purpose vaccine, next to enhancing the maximal epitope number, MHC-restriction of the encoded peptides has to be considered. All-in-one vaccines could be applied globally without being restricted by the vaccinees MHC-environment. Nevertheless, it remains unclear whether peptide enrichment is sufficient for the expansion of breadth and depth of the immune responses. Since protein structure and amino acid sequence strongly influence proteolysis as well as peptide translocation and presentation (see E.3.1.3,) not every single peptide theoretically present in an antigen is efficiently generated by the processing machinery for presentation on APC.
To analyze the breadth of antigen presentation on mdDC induced by the newly designed candidate vaccines, presentation of the HLA-A*02 restricted p17 SP9 (SLFNTVATP) peptide was analyzed. The SP9 peptide encoded in the 97CN54/001 isolate represents a variant of the well-known SL9 peptide (SLYNTVATL) [228,229] originated by immune escape mutations. Although SL9 was described to be immunodominant, SP9 presentation could not be detected. The absence of SP9 presentation could be attributed to (i) altered proteolysis of 97CN54/001 derived antigens triggered by escape mutations in the peptide itself or flanking regions, (ii) reduced SP9 translocation into the ER by the TAP-complex and (iii) a decrease in MHC binding affinities compared to SL9. Since SP9 specific CTL can be detected and isolated from HIV-1 positive donors with an HLA-A*02 environment, proteolysis and peptide presentation are not inhibited by the peptide itself. Certainly, protein folding or peptide flanking regions in the CN54 Gag might inhibit SP9 presentation efficiently. Because existence of SP9 specific CTL in the 97CN54 donor was not explored, there is no evidence of SP9 presentation elicited by this Gag variant.

In summary, it can be stated that epitope maximization does not necessarily result in an enhanced number of presented peptides. Since epitopes are found to cluster at distinct regions, efficient processing of one peptide might exclude formation of a partially overlapping epitope.

E.5 The artificial polyprotein GPN elicits only weak Gag-directed CTL responses in Balb/C mice

For analysis of immunogenicity, safety issues and optimal application routes, Balb/C mice were immunized with the NYVAC-based vaccine candidates. The illustrated data (see Fig. E.1) were kindly provided by Katharina Böckl (Molecular Microbiology and Gene Therapy Unit, Institute of Medical Microbiology and Hygiene, University of Regensburg, Head of Department Prof. Dr. Ralf Wagner)

Briefly, in Balb/c mice, deletion of gp120 did not result in enhanced Gag-specific T cell responses. However, enhanced Gag-expression elicited by reconstitution of the natural occurring frame shift induces enhanced $CD8^+$ T cell responses. Thus, in the BALB/c mice the immunogenicity of an antigen was strongly enhanced by increased Gag steady-state levels. Additional modifications such as size-reduction by PN-2deletion or VLP-formation did not further enhance the Gag-specific T Cell responses. Hence, in murine *in vivo* priming assays, immunogen steady-state levels mainly influence CTL restimulation. Assuming that the immunogens have similar CTL priming characteristics in mice and humans, the poor induction of Gag-specific CTL responses of NYVAC-C recorded in the EuroVacc studies is most likely not caused by expression of gp120. The low immunogenicity is rather due to the weak expression of the artificial GPN polyprotein.

The influence of direct and cross priming on generating CTL responses can not be evaluated in the murine *in vivo* priming assays. Since budding competence does not increase the Gag-specific $CD8^+$ T cell response, it can be concluded that cross presentation elicited by VLPs does not account for generating efficient immune responses.

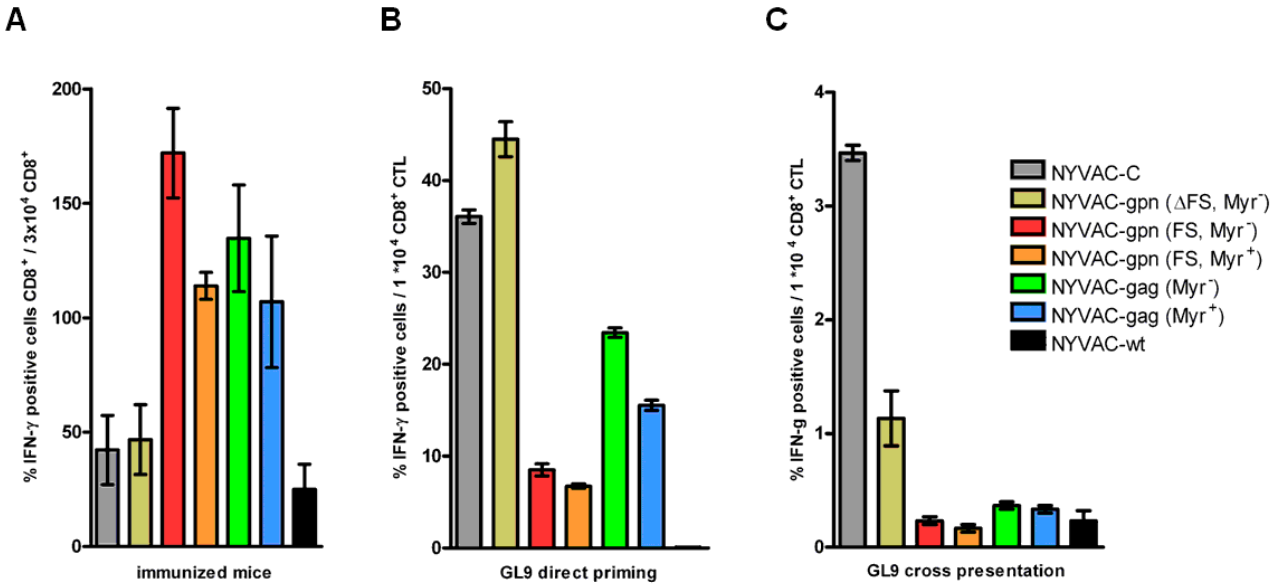


Fig. E.1 Illustration of the vaccine candidates' immunogenicity in *in vivo* mouse studies as well as *in vitro* antigen presentation assays utilizing human mdDC and GL9 CTL.

A, Six BALB/c mice (6 weeks old, Janvier) per group were immunized with $1x10^7$ NYVAC viruses. The vaccines were applied intramuscularly by injection into the *Tibialis anterior*. 14 days after immunization, splenocytes were isolated and restimulated by the $H2^d$ restricted A9I (AMQILKDTI) peptide. In an ICS, $CD8^+$ gated cells were analyzed for stimulation by detection of IFN-γ expression.

B, Human mdDC were infected with different transgenic NYVAC at MOI 5, incubated for 6 h and cocultivated with GL9 CTL. The cells were incubated for 10 h, CTL were analyzed for IFN-γ expression by ICS.

C, HeLa cells were infected with different transgenic NYVAC at MOI 5, incubated for 4 h, cocultivated with mdDC for 4 h, subsequently followed by GL9 CTL coculturing. The culture was maintained for 10 h. After incubation, $CD8^+$ CTL were analyzed for IFN-γ expression by ICS.

Importantly, the conclusions drawn from the mouse model are not in agreement with predictions made on the basis of human *in vitro* direct and cross presentation assays. In these assays, the GPN polyprotein induces best antigen presentation whereas antigen expression levels and VLP budding were shown to influence antigen presentation only slightly. The variations observed for both models could be attributed to (i) MHC type-dependent peptide presentation, (ii) variations in peptide presentation in murine and human APC, (iii) differences in TCR repertoires and thymic selection and (iv) discrepancies between T cell stimulation *in vivo* and restimulation *in vitro*.

As shown by the differences observed for antigen presentation of the HLA-B*07 restricted GL9 and HLA-A*02 SP9 peptides, the peptide component of the pMHC complex has a strong influence on CTL restimulation. Peptide presentation by certain MHC complexes was shown to induce protective CTL responses whereas other HLA-types failed to mediate protection (see B.2.2.2). Thus, the *ex vivo* stimulation assays of human CTL and murine the *in vivo* responses in the murine model are of limited comparability, because the HLA systems of both organisms show broad variations. Since the major part of the T cell receptors bind to HLA-subunits of the pMHC

complex, variations in the MHC environment and thymic selection between both species could induce differences in the T cell receptor repertoire. Thus, peptide presentation in mice and humans can differ among each other. The observed immune responses might be exclusive for Balb/C mice.

Due to differences in protein degradation depending on the proteasome composition, variations in peptide processing might be elicited by different proteolysis patterns of human und murine proteasomes [156,307]. Additionally, the TAP-dependent peptide translocation from the cytosol to the ER was shown to be peptide-specific and could vary between both species (see B.2.1.3.2).

Although differences in proteolysis, peptide translocation and presentation among mice and humans could alter an antigen's immunogenicity, variations are most likely elicited by differences between T cell stimulation *in vivo* and restimulation of CTL clones *in vitro*. *In vitro* priming of naïve $CD8^+$ T cells was described to be ineffective and requires artificial cell settings that do not correspond to the physiological priming in the lymph node [308,309]. Therefore, an *in vitro* assay utilizing already primed HIV-Gag specific T cell clones was applied. In contrast to CTL stimulation in *in vivo* assays, $CD4^+$ T helper cells are dispensable for *ex vivo* $CD8^+$ restimulation [310]. Hence, restimulation of specific CTL is not as restricted as activation of naive $CD8^+$ cells. Furthermore, CTL restimulation already occurs with lower numbers of pMHC complexes compared to stimulation. Thus, restimulation of CTL clones derived from HIV-1 infected donors can occur despite limited peptide presentation.
Additionally, in an *in vivo* assay, the viruses are not distributed equally among the cells next to the injection site. As shown for *ex vivo* direct and cross priming assays, the virus concentration strongly influences the character of peptide presentation by APC. Thus, differences observed between *in vivo* and *ex vivo* could be elicited by varying virus amounts.

In summary, extensive variations in the immune system between mice and humans hamper the comparability between both species. The human *ex vivo* assays allow analysis of the antigen's capacity to be presented on APC. Nevertheless, due to the non-physiological conditions, demonstrated antigen presentation does not necessarily result in increased immunogenicity.

E.6 Outlook

In direct and cross presentation assays, only low immunogenic capacities of physiologically shaped new generation Gag-variants were observed whereas the artificial polyprotein GPN induced extensive peptide presentation. In the clinical phase-I trial EuroVacc02, NYVAC-C (coding for GPN and gp120) was shown to elicit only weak Gag, Pol and Nef specific CTL-responses in humans. As shown, without co-administration of the gp120 protein direct presentation was significantly increased. Thus, *in vivo* application of NYVAC-gpn (ΔFS, Myr^-) could induce higher Gag, Pol and Nef specific CTL-responses in humans. Additionally, enhanced GPN-expression could amplify its immunogenicity in humans. Increased expression can be achieved by codon optimization or modifications of regulatory elements such as the insertion of a stronger promoter or enhancer elements. Since the expression of late poxviral gene products is inhibited in APC, poor expression could be circumvented by immunogen application with another vector system. Viral vectors such as Adenoviruses that infect APC could elicit direct presentation and therefore induce CTL responses more efficiently. Additionally, immune-escape mechanisms characteristic for NYVAC resulting in poor APC maturation could be avoided. More extensive APC maturation could result in increased peptide presentation and therefore enhance T cell priming. However, when applying alternative vector systems, (i) elicitation or side effects, (ii) pre-existing immune responses and (iii) immunodominance of vector-own antigens have to be excluded.

To enhance immunogenicity of HIV-1 Gag, variants with non-physiological protein shape leading to rapid degradation could be developed. By interrupting the native Gag-folding e.g. by scrambling particular subunits, proteasomal digestion might be enhanced. To detect Gag variants with optimal immunogenicity, a library of scrambled Gag-variants could be generated and screened in CTL-restimulation assays.

An alternative and promising approach to generate immunogens that induce broadly protective CTL is the development of so-called mosaic-antigens. Gag-based mosaic antigens are designed to contain a maximized number of epitopes that cover the HIV-1 sequence diversity [220]. At the Molecular Microbiology and Gene Therapy Unit of the University of Regensburg, a new bioinformatic algorithm for the design of epitope-enriched mosaic-antigens has been developed. By this algorithm, an array of Gag-based immunogens with user-defined characteristics can be designed. To determine antigen presentation capacity of such newly developed mosaic-antigens, they have to be screened for direct and cross presentation in a CTL-restimulation assay.

In a murine *in vivo* assay, enhanced Gag steady-state levels induced increased Gag-specific CTL responses whereas in an human *ex vivo* system antigen presentation could not be increased by an enhanced Gag expression-rate. Thus, to determine the validity of the results obtained with these assays, further studies that explain the discrepancies between both assays are required. Therefore, analysis of the new generation vaccine candidates in outbred mice with enhanced

genetic diversity together with a non-human primate study could demonstrate the relevance of the results obtained in this work. Detection of antigen presentation by restimulation of various CTL clones in a more extensive *ex vivo* assay could support the results obtained from *in vivo* studies.
A reliable method that simulates CTL activation in humans accurately could help to design immunogens more efficiently.

Although the newly designed immunogens analyzed in this study did not exhibit antigen presentation capacity to the desired degree, the results are likely to guide the rational design of Gag-based CTL vaccines. The consideration that a non-physiological shape of a protein strongly enhances its antigen presentation-rate should be the starting point for a next generation of antigens with increased immunogenic features.

F Appendix

F.1 Abbreviations

If not stated otherwise, abbreviations follow the recommendations from the Chicago Manual of Style [311], and biochemical abbreviations follow the Gold Book of IUPAC [312] and the list of common abbreviations by the Journal of Biological Chemistry [313].

97CN001	HIV-1 C-Clade provirus isolate
97CN54	HIV-1 C-Clade provirus isolate
A224	HIV-1 C-Clade gp120 isolate
Ad5	Adeno serotype 5
AI9	
AIDS	aquired immunodeficiency syndrome
AIDSVAX B/B	HIV-1 vaccine consisting of recombinant B- and C-Clade gp120
ALVAC	canarypox virus
APC	antigen presenting cell
APC-Cy7	allophycocyanin-cyanine7 dye
Balb/C	albino, laboratory-bred mouse strain
BFA	brefeldin A
BHK-21	baby hamster kidney cells
bp	base pairs
C57BL/6	inbred strain of laboratory mice
CCR	chemokine (C-C motif) receptor
IL	interleukine
CD	cluster of differentiation
CEV	cell-associated enveloped virions
CTL	cytotoxic T lymphocyte
CTLA	cytotoxic T-lymphocyte antigen
CXCR	C-X-C chemokine receptor
DC	dendritic cell
DF-1	chicken embryo fibroblast cell line
DMEM	Dulbecco's modified Eagle medium
DNA	deoxyribonucleic acid
dsRNA	double-stranded RNA
E/L	early/late
EBV	Epstein-Barr-Virus
EDTA	ethylenediaminetetraacetic acid
eIF-2a	eukaryotic translation initiation factor 2A
Env	HIV-1 envelope protein
ER	endoplasmic reticulum
ESCRT	endosomal sorting complex required for transport
EV	extracellular virions
EV01	EuroVacc01, HIV vaccine phase-I clinical trial
EV02	EuroVacc02, HIV vaccine phase-I clinical trial
FACS	fluorescence activated cell sorting
FasL	Fas ligand, type-II transmembrane protein that induces apoptosis
FCS	fetal calf serum
Fig	figure
FITC	Fluorescein isothiocyanate
FSC	forward scatter
Gag	group-specific antigen
GL9	HIV-1 Gag p24 derived peptide, GPGHKARVL
GM-CSF	granulocyte macrophage colony-stimulating factor
GNE8	GNE8 rgp120 C-Clade HIV-1 strain
gp	glycoprotein
GPN	artificial Gag-Pol-Nef polyprotein
HAART	highly active anti-retroviral therapy
HeLa	Henrietta Lacks, cervix carcinoma derived cell line
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
HLA-DR	MHC class II cell surface receptor allele
HRP	horseradish peroxidase
hpi	Hours post infection
ICS	intracellular cytokine staining
IFN	interferon
Ig	immunoglobulin
IL	interleukin, subgroup of cytokines
IN	HIV-1 integrase

IV	immature viral
LAI	B-Clade HIV-1 strain
LB	lysogeny broth
LCL	human B lymphoblastoid cell lines
LDC	Langerhans cell, mDC subpopulation
LCMV	Lymphocytic Choriomeningitis virus
LPS	lipopolysaccharides
LTR	long terminal repeat
Ma	HIV-1 matrix protein
MACS	magnetic-activated cell sorting
mDC	myeloid dendritic cell
mdDC	monocyte derived dendritic cell
MHC	major histocompatibility complex
MIP	macrophage inflammatory protein
MOI	multiplicity of infection
MRK-Ad5	AIDS vaccine candidate based on Ad5, developed by Merck KGaA
mRNA	messenger RNA
MTBS	TBS buffer compounded with 5% skim milk powder
MV	mature virion
MVA	modified vaccinia ankara
Myr	myristoylation
NC	nucleocapsid
Nef	negative regulatory factor
NFkB	nuclear factor 'kappa-light-chain-enhancer' of activated B-cells, transcription factor
NYVAC	New York Vaccinia, vP866
OD	optical density
ORF	open reading frame
PBS	phosphat buffered saline
pDC	plasmacytoid dendritic cell
PEST	proline, glutamic acid, serine, and threonine rich sequences
PHA-L	Phytohaemagglutinin agglutinating leukocytes
PIC	pre-initiation complex
pMHC	peptide-MHC complex
pMHC:TCR	peptide-MHC-TCR complex
Pol	HIV-1 polyprotein
PR	HIV-1 protease
Rev	HIV-1 regulator of virion
RIPA	radioimmunoprecipitation assay buffer
RNA	ribonucleic acid
RPMI	Roswell Park Memorial Institute medium
RRE	Rev-responsive element
RT	HIV-1 reverse transcriptase
RTC	reverse transcription complex
RV144	HIV vaccine phase-III clinical trial
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SL9	HIV-1 Gag p17 derived peptide, SLYNTVATL
snp	single nucleotide polymorphism
SOB	super optimal broth
SOC	SOB containing glucose
SP9	HIV-1 Gag p17 derived peptide, SLFNTVATP
SSC	side scatter
STI	structured treatment interruptions
Tab	table
TAP	Transporter associated with antigen processing
Tat	transactivator of transcription
TB	Transfomation buffer
TCA	trichloroacetic acid
TCGF	T cell growth factor
TCR	T cell receptor
TE	Tris-EDTA buffer
TLR	Toll-like receptor
wt	wild type

F.2 Characteristics and functions of surface molecules, chemokines and cytokines

Surface molecules and receptors

CCR5	chemokine (C-C motif) receptor, mediating inflammatory responses, expressed on T cells, DC and macrophages
CD4	coreceptor for T cell activation by TCRs, expressed on T cells
CD8	coreceptor for T cell activation by TCRs, expressed on T cells
CD14	monocyte surface marker, co-receptor for LPS detection
CD28	co-stimulatory molecule necessary for T cell activation, expressed on T cells
CD40	co-stimulatory molecule, expressed on APC
CD40L	CD40 ligand, expressed on T cells
CD80	also B7-1, co-stimulatory molecule necessary for T cell activation, expressed on monocytes
CD86	also B7-2, co-stimulatory molecule necessary for T cell activation, expressed on APC
CTLA4	cytotoxic T-lymphocyte antigen 4, transmits an inhibitory signal to T cells, expressed on T cells
CXCR4	C-X-C chemokine receptor type 4, chemotactic agent, expressed on T cells
DC-SIGN	dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin, C-type lectin receptor, expressed on DC
DC-LAMP	dendritic cell lysosome-associated membrane glycoprotein, expressed on DC

Chemokines and cytokines

IFN-a	type I class interferon, innate immune response modulator, expressed by leukocytes
IFN-g	type II class of interferon, innate and adaptive immune response regulator, expressed by lymphocytes and NK cells
IL-1	regulator of immune responses, inflammatory reactions
IL-15	stimulation and maintenance of cellular immune responses
IL-4	stimulation of activated B-cell and T-cell proliferation
MIP-1a	Chemokine (C-C motif) ligand 3, proinflammatory activities such as leukocyte chemotaxis, expressed by macrophages
MIP-1b	Chemokine (C-C motif) ligand 3, proinflammatory activities such as leukocyte chemotaxis, expressed by macrophages
MIP3a	Chemokine (C-C motif) ligand 20, proinflammatory activities such as lymphocyte chemotaxis, expressed by macrophages
RANTES	Regulated upon Activation, Normal T-cell Expressed, and Secreted, Chemokine (C-C motif) ligand 5, proinflammatory activities such as T cell chemotaxis

F.3 Danksagung

An erster Stelle möchte ich Prof. Dr. Ralf Wagner dafür danken, daß er es mir ermöglicht hat, meine Promotionsarbeit in seiner Arbeitsgruppe anzufertigen. Zusätzlich möchte ich mich für die fachliche Unterstützung bedanken, ohne die diese Arbeit nicht hätte durchgeführt werden können.

Ebenso herzlich möchte ich mich bei Prof. Dr. Stefan Dübel für die bereitwillige Betreuung meiner Doktorarbeit und dem Interesse an meiner Arbeit bedanken.

Ein besonderer Dank gilt Dr. Josef Köstler und Dr. Jens Wild, die mich sowohl fachlich als auch bei der Einarbeitung in neue Techniken unterstützt haben. Besonders hervorheben möchte ich ihre Ausdauer bei fachlichen Diskussionen.

Außerdem möchte ich Dr. Kathrin Kindsmüller und Katharina Böckl für die Bereitstellung der Vakzinekandidaten danken. Ohne den unermüdlichen Eifer bei der Erstellung der unterschiedlichen Varianten wäre die Anfertigung der Arbeit in dieser Form nicht möglich gewesen.

Des Weiteren möchte ich Dr. Josef Köstler, Dr. Jens Wild, Dr. Kathrin Kindsmüller, Dr. Benedikt Asbach und Bradford Lefoley danken, die ihre Freizeit geopfert haben, um mir mit Verbesserungen bezüglich der Niederschrift meiner Promotionsarbeit behilflich zu sein.

Der gesamten AG Wagner möchte für die wirklich einzigartige Arbeitsatmosphäre danken. Ohne eure fachliche und private Unterstützung wäre meine Zeit in Regensburg sicherlich nicht so angenehm verlaufen, wie ich sie jetzt empfinde. Besonders unsere unzähligen „Get Together“ im Sozialflur werden mir unvergeßlich bleiben. Im Speziellen möchte ich noch meine Mitstreiterinnen und Andi im Labor Nr. 66 und 80 erwähnen. Ich danke euch für die bereitwillige (!) Aufnahme in euren bescheidenen Räumlichkeiten und den schönen Stunden, die wir miteinander verbringen konnten.

Dir, liebe Anne, danke ich dafür, dass du geduldig die örtliche Differenz von teilweise über 500 km zwischen uns ertragen hast und mich trotz allem dazu ermutigt hast, meine Promotion anzufertigen.

Ein besonderer Dank gilt meiner Familie, die mich neben sowohl persönlich als auch finanziell sehr stark unterstützt hat. Danke, dass ihr immer fest an mich geglaubt und mir Halt und Kraft gegeben habt.

F.4 Reference List

1. Barré-Sinoussi F, Chermann JC, Rey F, *et al.* Isolation of a T-lymphotropic retrovirus from a patient at risk for acquired immune deficiency syndrome (AIDS). *Science*. 1983;220(4599):868-871.

2. Parliament Office of Science and Technology. HIV/AIDS in developing countries. 2003.

3. Granich R, Crowley S, Vitoria M, *et al.* Highly active antiretroviral treatment as prevention of HIV transmission: review of scientific evidence and update. *Curr Opin HIV AIDS*. 2010;5(4):298-304.

4. Auvert B, Males S, Puren A, *et al.* Can highly active antiretroviral therapy reduce the spread of HIV?: A study in a township of South Africa. *J. Acquir. Immune Defic. Syndr*. 2004;36(1):613-621.

5. UNAIDS, UNFPA and WHO. Condoms and HIV prevention: Position statement by UNAIDS, UNFPA and WHO. 2009.

6. Anderson RM, Medley GF, May RM, Johnson AM. A preliminary study of the transmission dynamics of the human immunodeficiency virus (HIV), the causative agent of AIDS. *IMA J Math Appl Med Biol*. 1986;3(4):229-263.

7. Peterman TA, Drotman DP, Curran JW. Epidemiology of the acquired immunodeficiency syndrome (AIDS). *Epidemiol Rev*. 1985;7:1-21.

8. Plantier J-C, Leoz M, Dickerson JE, *et al.* A new human immunodeficiency virus derived from gorillas. *Nat Med*. 2009;15(8):871-872.

9. Hemelaar J, Gouws E, Ghys PD, Osmanov S. Global and regional distribution of HIV-1 genetic subtypes and recombinants in 2004. *AIDS*. 2006;20(16):W13-W23.

10. Takeb E Y, Kusagawa S, Motomura K. Molecular epidemiology of HIV: tracking AIDS pandemic. *Pediatr Int*. 2004;46(2):236-244.

11. Knipe D, Howley P, Griffin D. Fields Virology 2 Vol Set. Parental Adviso. 2006. Lippincott Williams & Wilkins.

12. Freed EO. HIV-1 replication. *Somat. Cell Mol. Genet.* 2001;26(1-6):13-33.

13. Frankel AD, Young JA. HIV-1: fifteen proteins and an RNA. *Annu. Rev. Biochem*. 1998;67:1-25.

14. Gelderblom HR, Özel M, Hausmann EHS, *et al.* Fine structure of human immunodeficiency virus (HIV), immunolocalization of structural proteins and virus-cell relation. *Micron and Microscopica Acta*. 1988;19(1):41-60.

15. Chertova E, Bess JW Jr, Crise BJ, *et al.* Envelope glycoprotein incorporation, not shedding of surface envelope glycoprotein (gp120/SU), is the primary determinant of SU content of purified human immunodeficiency virus type 1 and simian immunodeficiency virus. *J. Virol*. 2002;76(11):5315-5325.

16. Hoffmann C, Rockstroh J. *HIV 2010*.

17. Parkin NT, Chamorro M, Varmus HE. Human immunodeficiency virus type 1 gag-pol frameshifting is dependent on downstream mRNA secondary structure: demonstration by expression in vivo. *J Virol*. 1992;66(8):5147-5151.

18. Staple DW, Butcher SE. Solution structure of the HIV‐1 frameshift inducing stem–loop RNA. *Nucleic Acids Research*. 2003;31(15):4326 -4331.

19. Morikawa Y, Hinata S, Tomoda H, *et al.* Complete Inhibition of Human Immunodeficiency Virus Gag Myristoylation is Necessary for Inhibition of Particle Budding. *Journal of Biological Chemistry*. 1996;271(5):2868 -2873.

20. Pal R, Reitz MS Jr, Tschachler E, *et al.* Myristoylation of Gag proteins of HIV-1 plays an important role in virus assembly. *AIDS Res. Hum. Retroviruses*. 1990;6(6):721-730.

21. Provitera P, El-Maghrabi R, Scarlata S. The effect of HIV-1 Gag myristoylation on membrane binding. *Biophysical Chemistry*. 2006;119(1):23-32.

22. Wang JK, Kiyokawa E, Verdin E, Trono D. The Nef protein of HIV-1 associates with rafts and primes T cells for activation. *Proc. Natl. Acad. Sci. U.S.A*. 2000;97(1):394-399.

23. Gorelick RJ, Nigida SM Jr, Bess JW Jr, *et al.* Noninfectious human immunodeficiency virus type 1 mutants deficient in genomic RNA. *J. Virol*. 1990;64(7):3207-3211.

24. Office of the Technology Assessment. *Review of the Public Health Service's response to AIDS*. 1985.

25. International AIDS Vaccine Initiative. AVI Report Database of AIDS Vaccine Candidates in Clinical Trials. 2011.

26. Gilbert PB, Berger JO, Stablein D, *et al.* Statistical Interpretation of the RV144 HIV Vaccine Efficacy Trial in Thailand: A Case Study for Statistical Issues in Efficacy Trials. *Journal of Infectious Diseases*. 2011;203(7):969 -975.

27. Rerks-Ngarm S, Pitisuttithum P, Nitayaphan S, *et al.* Vaccination with ALVAC and AIDSVAX to prevent HIV-1 infection in Thailand. *N. Engl. J. Med*. 2009;361(23):2209-2220.

28. Wolfs TF, de Jong JJ, Van den Berg H, *et al.* Evolution of sequences encoding the principal neutralization epitope of human immunodeficiency virus 1 is host dependent, rapid, and continuous. *Proc. Natl. Acad. Sci. U.S.A*. 1990;87(24):9938-9942.

29. Perelson AS, Ribeiro RM. Estimating drug efficacy and viral dynamic parameters: HIV and HCV. *Statistics in Medicine*. 2008;27(23):4647-4657.

30. Bebenek K, Abbotts J, Wilson SH, Kunkel TA. Error-prone polymerization by HIV-1 reverse transcriptase. Contribution of template-primer misalignment, miscoding, and termination probability to mutational hot spots. *Journal of Biological Chemistry*. 1993;268(14):10324 -10334.

31. Gnanakaran S, Lang D, Daniels M, *et al.* Clade-specific differences between human immunodeficiency virus type 1 clades B and C: diversity and correlations in C3-V4 regions of gp120. *J. Virol*. 2007;81(9):4886-4891.

32. Lal RB, Chakrabarti S, Yang C. Impact of genetic diversity of HIV-1 on diagnosis, antiretroviral therapy & vaccine development. *Indian J. Med. Res*. 2005;121(4):287-314.

33. Mthunzi P, Meyer D. Limited cross-reactivity between different HIV-1 clades. *Journal of Clinical Virology*. 2004;31(Supplement 1):88-91.

34. Reitter JN, Means RE, Desrosiers RC. A role for carbohydrates in immune evasion in AIDS. *Nat Med*. 1998;4(6):679-684.

35. Zhu P, Chertova E, Bess J, *et al.* Electron tomography analysis of envelope glycoprotein trimers on HIV and simian immunodeficiency virus virions. *Proceedings of the National Academy of Sciences*. 2003;100(26):15812 -15817.

36. Kwong PD, Doyle ML, Casper DJ, *et al.* HIV-1 evades antibody-mediated neutralization through conformational masking of receptor-binding sites. *Nature*. 2002;420(6916):678-682.

37. Girard MP, Osmanov SK, Kieny MP. A review of vaccine research and development: The human immunodeficiency virus (HIV). *Vaccine*. 2006;24(19):4062-4081.

38. Hoxie JA. Toward an antibody-based HIV-1 vaccine. *Annu. Rev. Med*. 2010;61:135-152.

39. Poon B, Hsu JF, Gudeman V, Chen ISY, Grovit-Ferbas K. Formaldehyde-Treated, Heat-Inactivated Virions with Increased Human Immunodeficiency Virus Type 1 Env Can Be Used To Induce High-Titer Neutralizing Antibody Responses. *J. Virol.* 2005;79(16):10210-10217.

40. de Rosny E, Vassell R, Jiang S, Kunert R, Weiss CD. Binding of the 2F5 Monoclonal Antibody to Native and Fusion-Intermediate Forms of Human Immunodeficiency Virus Type 1 gp41: Implications for Fusion-Inducing Conformational Changes. *J. Virol.* 2004;78(5):2627-2631.

41. Cardoso RMF, Zwick MB, Stanfield RL, *et al.* Broadly neutralizing anti-HIV antibody 4E10 recognizes a helical conformation of a highly conserved fusion-associated motif in gp41. *Immunity*. 2005;22(2):163-173.

42. Bojak A, Deml L, Wagner R. The past, present and future of HIV-vaccine development: a critical view. *Drug Discovery Today*. 2002;7(1):36-46.

43. Pauza CD. HIV persistence in monocytes leads to pathogenesis and AIDS. *Cell. Immunol*. 1988;112(2):414-424.

44. Borrow P, Lewicki H, Hahn BH, Shaw GM, Oldstone MB. Virus-specific CD8+ cytotoxic T-lymphocyte activity associated with control of viremia in primary human immunodeficiency virus type 1 infection. *J. Virol*. 1994;68(9):6103-6110.

45. Koup RA, Safrit JT, Cao Y, *et al.* Temporal association of cellular immune responses with the initial control of viremia in primary human immunodeficiency virus type 1 syndrome. *J. Virol*. 1994;68(7):4650-4655.

46. Schmitz JE, Kuroda MJ, Santra S, *et al.* Control of viremia in simian immunodeficiency virus infection by CD8+ lymphocytes. *Science*. 1999;283(5403):857-860.

47. Harrer T, Harrer E, Kalams SA, *et al.* Strong cytotoxic T cell and weak neutralizing antibody responses in a subset of persons with stable nonprogressing HIV type 1 infection. *AIDS Res. Hum. Retroviruses*. 1996;12(7):585-592.

48. Harrer T, Harrer E, Kalams SA, *et al.* Cytotoxic T lymphocytes in asymptomatic long-term nonprogressing HIV-1 infection. Breadth and specificity of the response and relation to in vivo viral quasispecies in a person with prolonged infection and low viral load. *J. Immunol*. 1996;156(7):2616-2623.

49. Rowland-Jones S, Sutton J, Ariyoshi K, *et al.* HIV-specific cytotoxic T-cells in HIV-exposed but uninfected Gambian women. *Nat Med*. 1995;1(1):59-64.

50. Sacha JB, Chung C, Rakasz EG, *et al.* Gag-specific CD8+ T lymphocytes recognize infected cells before AIDS-virus integration and viral protein expression. *J. Immunol*. 2007;178(5):2746-2754.

51. Collins KL. Resistance of HIV-infected cells to cytotoxic T lymphocytes. *Microbes Infect*. 2004;6(5):494-500.

52. Collins KL. How HIV evades CTL recognition. *Curr. HIV Res*. 2003;1(1):31-40.

53. Carrington M, O'Brien SJ. The influence of HLA genotype on AIDS. *Annu. Rev. Med*. 2003;54:535-551.

54. Ogg GS, Jin X, Bonhoeffer S, *et al.* Quantitation of HIV-1-Specific Cytotoxic T Lymphocytes and Plasma Load of Viral RNA. *Science*. 1998;279(5359):2103 -2106.

55. Letvin NL. Strategies for an HIV vaccine. *J. Clin. Invest.* 2002;110(1):15-27.

56. Murphey-Corb M. Live-attenuated HIV vaccines: How safe is safe enough? *Nat Med*. 1997;3(1):17-18.

57. Sauter SL, Rahman A, Muralidhar G. Non-replicating viral vector-based AIDS vaccines: interplay between viral vectors and the immune system. *Curr. HIV Res*. 2005;3(2):157-181.

58. Marovich MA. ALVAC-HIV vaccines: clinical trial experience focusing on progress in vaccine development. *Expert Rev Vaccines*. 2004;3(4 Suppl):S99-104.

59. Pantaleo G, Esteban M, Jacobs B, Tartaglia J. Poxvirus vector-based HIV vaccines. *Curr Opin HIV AIDS*. 2010;5(5):391-396.

60. Priddy FH, Brown D, Kublin J, *et al.* Safety and immunogenicity of a replication-incompetent adenovirus type 5 HIV-1 clade B gag/pol/nef vaccine in healthy adults. *Clin. Infect. Dis*. 2008;46(11):1769-1781.

61. Catanzaro AT, Koup RA, Roederer M, *et al.* Phase 1 Safety and Immunogenicity Evaluation of a Multiclade HIV‐1 Candidate Vaccine Delivered by a Replication‐Defective Recombinant Adenovirus Vector. *Journal of Infectious Diseases*. 2006;194(12):1638 -1649.

62. Buchbinder SP, Mehrotra DV, Duerr A, *et al.* Efficacy assessment of a cell-mediated immunity HIV-1 vaccine (the Step Study): a double-blind, randomised, placebo-controlled, test-of-concept trial. *Lancet*. 2008;372(9653):1881-1893.

63. McElrath MJ, De Rosa SC, Moodie Z, *et al.* HIV-1 vaccine-induced immunity in the test-of-concept Step Study: a case-cohort analysis. *Lancet*. 2008;372(9653):1894-1905.

64. Perreau M, Pantaleo G, Kremer EJ. Activation of a dendritic cell–T cell axis by Ad5 immune complexes creates an improved environment for replication of HIV in T cells. *The Journal of Experimental Medicine*. 2008;205(12):2717 -2725.

65. Benlahrech A, Harris J, Meiser A, *et al.* Adenovirus vector vaccination induces expansion of memory CD4 T cells with a mucosal homing phenotype that are readily susceptible to HIV-1. *Proc. Natl. Acad. Sci. U.S.A*. 2009;106(47):19940-19945.

66. O A. HIV gp120 vaccine - VaxGen: AIDSVAX, AIDSVAX B/B, AIDSVAX B/E, HIV gp120 vaccine - Genentech, HIV gp120 vaccine AIDSVAX - VaxGen, HIV vaccine AIDSVAX - VaxGen. *Drugs R D*. 2003;4(4):249-253.

67. Goebel SJ, Johnson GP, Perkus ME, *et al.* The complete DNA sequence of vaccinia virus. *Virology*. 1990;179(1):247-266.

68. Moss B. Poxvirus entry and membrane fusion. *Virology*. 2006;344(1):48-54.

69. Broyles SS. Vaccinia virus transcription. *J. Gen. Virol*. 2003;84(Pt 9):2293-2303.

70. Condit RC. Vaccinia, Inc.—Probing the Functional Substructure of Poxviral Replication Factories. *Cell Host & Microbe*. 2007;2(4):205-207.

71. Smith GL, Vanderplasschen A, Law M. The formation and function of extracellular enveloped vaccinia virus. *Journal of General Virology*. 2002;83(12):2915 -2931.

72. Smith GL, Law M. The exit of vaccinia virus from infected cells. *Virus Res*. 2004;106(2):189-197.

73. Collier LH. The development of a stable smallpox vaccine. *The Journal of hygiene*. 53(1):76-101.

74. Pastoret P-P, Vanderplasschen A. Poxviruses as vaccine vectors. *Comp. Immunol. Microbiol. Infect. Dis*.

2003;26(5-6):343-355.

75. Gherardi MM, Esteban M. Recombinant poxviruses as mucosal vaccine vectors. *J. Gen. Virol*. 2005;86(Pt 11):2925-2936.

76. Mooij P, Balla-Jhagjhoorsingh SS, Koopman G, *et al.* Differential CD4+ versus CD8+ T-cell responses elicited by different poxvirus-based human immunodeficiency virus type 1 vaccine candidates provide comparable efficacies in primates. *J. Virol*. 2008;82(6):2975-2988.

77. Cono J, Casey C, Bell D. Smallpox Vaccination and Adverse Reactions. 2003. Available at: http://www.cdc.gov/mmwr/preview/mmwrhtml/rr5204a1.htm

78. Tartaglia J, Perkus ME, Taylor J, *et al.* NYVAC: a highly attenuated strain of vaccinia virus. *Virology*. 1992;188(1):217-232.

79. Guerra S, Nájera JL, González JM, *et al.* Distinct gene expression profiling after infection of immature human monocyte-derived dendritic cells by the attenuated poxvirus vectors MVA and NYVAC. *J. Virol*. 2007;81(16):8707-8721.

80. Guerra S, López-Fernández LA, Pascual-Montano A, *et al.* Host Response to the Attenuated Poxvirus Vector NYVAC: Upregulation of Apoptotic Genes and NF-κB-Responsive Genes in Infected HeLa Cells. *J Virol*. 2006;80(2):985-998.

81. Nájera JL, Gómez CE, Domingo-Gil E, Gherardi MM, Esteban M. Cellular and biochemical differences between two attenuated poxvirus vaccine candidates (MVA and NYVAC) and role of the C7L gene. *J. Virol*. 2006;80(12):6033-6047.

82. Paoletti E. Applications of pox virus vectors to vaccination: an update. *Proceedings of the National Academy of Sciences*. 1996;93(21):11349 -11353.

83. Engelmayer J, Larsson M, Subklewe M, *et al.* Vaccinia Virus Inhibits the Maturation of Human Dendritic Cells: A Novel Mechanism of Immune Evasion. *The Journal of Immunology*. 1999;163(12):6762 -6768.

84. Seet BT, Johnston JB, Brunetti CR, *et al.* Poxviruses and immune evasion. *Annu. Rev. Immunol*. 2003;21:377-423.

85. McCormack S, Stöhr W, Barber T, *et al.* EV02: a Phase I trial to compare the safety and immunogenicity of HIV DNA-C prime-NYVAC-C boost to NYVAC-C alone. *Vaccine*. 2008;26(25):3162-3174.

86. Quakkelaar ED, Redeker A, Haddad EK, *et al.* Improved Innate and Adaptive Immunostimulation by Genetically Modified HIV-1 Protein Expressing NYVAC Vectors. *PLoS ONE*. 2011;6(2):e16819.

87. Gómez CE, Nájera JL, Jiménez EP, *et al.* Head-to-head comparison on the immunogenicity of two HIV/AIDS vaccine candidates based on the attenuated poxvirus strains MVA and NYVAC co-expressing in a single locus the HIV-1BX08 gp120 and HIV-1(IIIB) Gag-Pol-Nef proteins of clade B. *Vaccine*. 2007;25(15):2863-2885.

88. Harari A, Bart P-A, Stöhr W, *et al.* An HIV-1 clade C DNA prime, NYVAC boost vaccine regimen induces reliable, polyfunctional, and long-lasting T cell responses. *J. Exp. Med*. 2008;205(1):63-77.

89. Gómez CE, Nájera JL, Krupa M, Esteban M. The poxvirus vectors MVA and NYVAC as gene delivery systems for vaccination against infectious diseases and cancer. *Curr Gene Ther*. 2008;8(2):97-120.

90. Barry M, Bleackley RC. Cytotoxic T lymphocytes: all roads lead to death. *Nat Rev Immunol*. 2002;2(6):401-409.

91. Purbhoo MA, Boulter JM, Price DA, *et al.* The Human CD8 Coreceptor Effects Cytotoxic T Cell Activation and Antigen Sensitivity Primarily by Mediating Complete Phosphorylation of the T Cell Receptor ζ Chain. *Journal of*

Biological Chemistry. 2001;276(35):32786 -32792.

92. Slavik JM, Hutchcroft JE, Bierer BE. CD28/CTLA-4 and CD80/CD86 families: signaling and function. *Immunol. Res*. 1999;19(1):1-24.

93. Trapani JA. Target cell apoptosis induced by cytotoxic T cells and natural killer cells involves synergy between the pore-forming protein, perforin, and the serine protease, granzyme B. *Aust N Z J Med*. 1995;25(6):793-799.

94. Shresta S, Pham CT, Thomas DA, Graubert TA, Ley TJ. How do cytotoxic lymphocytes kill their targets? *Curr. Opin. Immunol*. 1998;10(5):581-587.

95. Pantaleo G, Graziosi C, Demarest JF, *et al.* Role of lymphoid organs in the pathogenesis of human immunodeficiency virus (HIV) infection. *Immunol. Rev*. 1994;140:105-130.

96. Shortman K, Liu Y-J. Mouse and human dendritic cell subtypes. *Nat. Rev. Immunol*. 2002;2(3):151-161.

97. Steinman RM. The dendritic cell system and its role in immunogenicity. *Annu. Rev. Immunol*. 1991;9:271-296.

98. Liu YJ. Dendritic cell subsets and lineages, and their functions in innate and adaptive immunity. *Cell*. 2001;106(3):259-262.

99. Romani N, Gruner S, Brang D, *et al.* Proliferating dendritic cell progenitors in human blood. *J. Exp. Med*. 1994;180(1):83-93.

100. Geijtenbeek TB, Torensma R, van Vliet SJ, *et al.* Identification of DC-SIGN, a novel dendritic cell-specific ICAM-3 receptor that supports primary immune responses. *Cell*. 2000;100(5):575-585.

101. Sallusto F, Lanzavecchia A. Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor alpha. *J. Exp. Med*. 1994;179(4):1109-1118.

102. Sallusto F, Cella M, Danieli C, Lanzavecchia A. Dendritic cells use macropinocytosis and the mannose receptor to concentrate macromolecules in the major histocompatibility complex class II compartment: downregulation by cytokines and bacterial products. *J. Exp. Med*. 1995;182(2):389-400.

103. Engering AJ, Cella M, Fluitsma D, *et al.* The mannose receptor functions as a high capacity and broad specificity antigen receptor in human dendritic cells. *Eur. J. Immunol*. 1997;27(9):2417-2425.

104. Albert ML, Sauter B, Bhardwaj N. Dendritic cells acquire antigen from apoptotic cells and induce class I-restricted CTLs. *Nature*. 1998;392(6671):86-89.

105. Rescigno M, Granucci F, Citterio S, Foti M, Ricciardi-Castagnoli P. Coordinated events during bacteria-induced DC maturation. *Immunol. Today*. 1999;20(5):200-203.

106. Satthaporn S, Eremin O. Dendritic cells (I): Biological functions. *J R Coll Surg Edinb*. 2001;46(1):9-19.

107. Dieu MC, Vanbervliet B, Vicari A, *et al.* Selective recruitment of immature and mature dendritic cells by distinct chemokines expressed in different anatomic sites. *J. Exp. Med*. 1998;188(2):373-386.

108. Nelson D, Thomas JA, Holt PG, McWilliam AS. Rapid dendritic cell recruitment is a hallmark of the acute inflammatory response at mucosal surfaces. *The Journal of Experimental Medicine*. 179(4):1331-1336.

109. Power CA, Church DJ, Meyer A, *et al.* Cloning and Characterization of a Specific Receptor for the Novel CC Chemokine MIP-3α from Lung Dendritic Cells. *The Journal of Experimental Medicine*. 1997;186(6):825 -835.

110. Tang A, Amagai M, Granger LG, Stanley JR, Udey MC. Adhesion of epidermal Langerhans cells to keratinocytes mediated by E-cadherin. *Nature*. 1993;361(6407):82-85.

111. Burns S, Hardy SJ, Buddle J, *et al.* Maturation of DC is associated with changes in motile characteristics and adherence. *Cell Motil. Cytoskeleton*. 2004;57(2):118-132.

112. Lindquist RL, Shakhar G, Dudziak D, *et al.* Visualizing dendritic cell networks in vivo. *Nat Immunol*. 2004;5(12):1243-1250.

113. Ngo VN, Tang HL, Cyster JG. Epstein-Barr virus-induced molecule 1 ligand chemokine is expressed by dendritic cells in lymphoid tissues and strongly attracts naive T cells and activated B cells. *J. Exp. Med*. 1998;188(1):181-191.

114. McLellan AD, Sorg RV, Williams LA, Hart DN. Human dendritic cells activate T lymphocytes via a CD40: CD40 ligand-dependent pathway. *Eur. J. Immunol*. 1996;26(6):1204-1210.

115. Moore MW, Carbone FR, Bevan MJ. Introduction of soluble protein into the class I pathway of antigen processing and presentation. *Cell*. 1988;54(6):777-785.

116. Goldberg AL, St. John AC. Intracellular Protein Degradation in Mammalian and Bacterial Cells: Part 2. *Annu. Rev. Biochem.* 1976;45(1):747-804.

117. Hershko A, Ciechanover A. The Ubiquitin System for Protein Degradation. *Annu. Rev. Biochem.* 1992;61(1):761-807.

118. Palombella VJ, Rando OJ, Goldberg AL, Maniatis T. The ubiquitinproteasome pathway is required for processing the NF-κB1 precursor protein and the activation of NF-[kappa]B. *Cell*. 1994;78(5):773-785.

119. Yewdell JW, Bennink JR. Cell biology of antigen processing and presentation to major histocompatibility complex class I molecule-restricted T lymphocytes. *Adv. Immunol*. 1992;52:1-123.

120. Townsend A, Trowsdale J. The transporters associated with antigen presentation. *Seminars in Cell Biology*. 1993;4(1):53-61.

121. Xu R-H, Remakus S, Ma X, Roscoe F, Sigal LJ. Direct Presentation Is Sufficient for an Efficient Anti-Viral CD8+ T Cell Response. *PLoS Pathog*. 2010;6(2):e1000768.

122. de Saint-Vis B, Vincent J, Vandenabeele S, *et al.* A novel lysosome-associated membrane glycoprotein, DC-LAMP, induced upon DC maturation, is transiently expressed in MHC class II compartment. *Immunity*. 1998;9(3):325-336.

123. Engering AJ, Cella M, Fluitsma DM, *et al.* Mannose receptor mediated antigen uptake and presentation in human dendritic cells. *Adv. Exp. Med. Biol*. 1997;417:183-187.

124. Inaba K, Turley S, Yamaide F, *et al.* Efficient presentation of phagocytosed cellular fragments on the major histocompatibility complex class II products of dendritic cells. *J. Exp. Med*. 1998;188(11):2163-2173.

125. Morrison LA, Lukacher AE, Braciale VL, Fan DP, Braciale TJ. Differences in antigen presentation to MHC class I- and class II-restricted influenza virus-specific cytolytic T lymphocyte clones. *The Journal of Experimental Medicine*. 1986;163(4):903 -921.

126. Bevan MJ. Cross-priming for a secondary cytotoxic response to minor H antigens with H-2 congenic cells which do not cross-react in the cytotoxic assay. 1976. *J. Immunol*. 1976;185(3):1361-1366.

127. Bevan MJ. Priming for a cytotoxic response to minor histocompatibility antigens: antigen specificity and failure to demonstrate a carrier effect. *J. Immunol*. 1977;118(4):1370-1374.

128. den Haan JM, Lehar SM, Bevan MJ. CD8(+) but not CD8(-) dendritic cells cross-prime cytotoxic T cells in vivo. *J. Exp. Med*. 2000;192(12):1685-1696.

129. Mitchell DA, Nair SK, Gilboa E. Dendritic cell/macrophage precursors capture exogenous antigen for MHC class I presentation by dendritic cells. *Eur. J. Immunol*. 1998;28(6):1923-1933.

130. Huang AY., Bruce AT, Pardoll DM, Levitsky HI. In Vivo Cross-Priming of MHC Class I–Restricted Antigens Requires the TAP Transporter. *Immunity*. 1996;4(4):349-355.

131. Yewdell JW, Norbury CC, Bennink JR. Mechanisms of exogenous antigen presentation by MHC class I molecules in vitro and in vivo: implications for generating CD8+ T cell responses to infectious agents, tumors, transplants, and vaccines. *Adv. Immunol*. 1999;73:1-77.

132. Sigal LJ, Crotty S, Andino R, Rock KL. Cytotoxic T-cell immunity to virus-infected non-haematopoietic cells requires presentation of exogenous antigen. *Nature* 398, 77—80; 1999

133. Kurts C, Carbone FR, Barnden M, *et al.* CD4+ T Cell Help Impairs CD8+ T Cell Deletion Induced by Cross-presentation of Self-Antigens and Favors Autoimmunity. *The Journal of Experimental Medicine*. 1997;186(12):2057 -2062.

134. Pfeifer JD, Wick MJ, Roberts RL, *et al.* Phagocytic processing of bacterial antigens for class I MHC presentation to T cells. *Nature*. 1993;361(6410):359-362.

135. Kovacsovics-Bankowski M, Rock K. A phagosome-to-cytosol pathway for exogenous antigens presented on MHC class I molecules. *Science*. 1995;267(5195):243 -246.

136. Fonteneau JF, Larsson M, Bhardwaj N. Interactions between dead cells and dendritic cells in the induction of antiviral CTL responses. *Curr. Opin. Immunol*. 2002;14(4):471-477.

137. Nouri-Shirazi M, Banchereau J, Bell D, *et al.* Dendritic Cells Capture Killed Tumor Cells and Present Their Antigens to Elicit Tumor-Specific Immune Responses. *The Journal of Immunology*. 2000;165(7):3797 -3803.

138. Zitvogel L, Regnault A, Lozier A, *et al.* Eradication of established murine tumors using a novel cell-free vaccine: dendritic cell derived exosomes. *Nat Med*. 1998;4(5):594-600.

139. Serna A, Ramirez MC, Soukhanova A, Sigal LJ. Cutting Edge: Efficient MHC Class I Cross-Presentation during Early Vaccinia Infection Requires the Transfer of Proteasomal Intermediates between Antigen Donor and Presenting Cells. *The Journal of Immunology*. 2003;171(11):5668 -5672.

140. Ma X, Serna A, Xu R-H, Sigal LJ. The amino acids sequences flanking an antigenic determinant can strongly affect MHC class I cross-presentation without altering direct-presentation. *J Immunol*. 2009;182(8):4601-4607.

141. Heath WR, Carbone FR. Cross-presentation in viral immunity and self-tolerance. *Nat. Rev. Immunol.* 2001;1(2):126-134.

142. Gasteiger G, Kastenmuller W, Ljapoci R, Sutter G, Drexler I. Cross-Priming of Cytotoxic T Cells Dictates Antigen Requisites for Modified Vaccinia Virus Ankara Vector Vaccines. *J. Virol.* 2007;81(21):11925-11936.

143. Finley D, Chau V. Ubiquitination. *Annu. Rev. Cell. Biol.* 1991;7(1):25-69.

144. Varshavsky A. The N-end rule. *Cell*. 1992;69(5):725-735.

145. Wu Y, Kipps TJ. Deoxyribonucleic acid vaccines encoding antigens with rapid proteasome-dependent degradation are highly efficient inducers of cytolytic T lymphocytes. *J. Immunol*. 1997;159(12):6037-6043.

146. Tobery TW, Siliciano RF. Targeting of HIV-1 Antigens for Rapid Intracellular Degradation Enhances Cytotoxic T Lymphocyte (CTL) Recognition and the Induction of De Novo CTL Responses In Vivo after Immunization. *The Journal of Experimental Medicine*. 1997;185(5):909 -920.

147. Ciechanover A. The ubiquitin-proteasome proteolytic pathway. *Cell*. 1994;79(1):13-21.

148. Orlowski M. The multicatalytic proteinase complex, a major extralysosomal proteolytic system. *Biochemistry*. 1990;29(45):10289-10297.

149. Gaczynska M, Rock KL, Spies T, Goldberg AL. Peptidase activities of proteasomes are differentially regulated by the major histocompatibility complex-encoded genes for LMP2 and LMP7. *Proceedings of the National Academy of Sciences*. 1994;91(20):9213 -9217.

150. Orlowski M, Cardozo C, Michaud C. Evidence for the presence of five distinct proteolytic components in the pituitary multicatalytic proteinase complex. Properties of two components cleaving bonds on the carboxyl side of branched chain and small neutral amino acids. *Biochemistry*. 1993;32(6):1563-1572.

151. Bogyo M, Shin S, McMaster JS, Ploegh HL. Substrate binding and sequence preference of the proteasome revealed by active-site-directed affinity probes. *Chem. Biol*. 1998;5(6):307-320.

152. Varshavsky A. Naming a targeting signal. *Cell*. 1991;64(1):13-15.

153. Rechsteiner M, Rogers SW. PEST sequences and regulation by proteolysis. *Trends Biochem. Sci.* 1996;21(7):267-271.

154. Del Val M, Schlicht H-J, Ruppert T, Reddehase MJ, Koszinowski UH. Efficient processing of an antigenic sequence for presentation by MHC class I molecules depends on its neighboring residues in the protein. *Cell*. 1991;66(6):1145-1153.

155. Eisenlohr LC, Yewdell JW, Bennink JR. Flanking sequences influence the presentation of an endogenously synthesized peptide to cytotoxic T lymphocytes. *J. Exp. Med*. 1992;175(2):481-487.

156. Gaczynska M, Rock KL, Goldberg AL. γ-Interferon and expression of MHC genes regulate peptide hydrolysis by proteasomes. *Nature*. 1993;365(6443):264-267.

157. Cardozo C, Kohanski RA. Altered Properties of the Branched Chain Amino Acid-preferring Activity Contribute to Increased Cleavages after Branched Chain Residues by the “Immunoproteasome”. *Journal of Biological Chemistry*. 1998;273(27):16764 -16770.

158. Colonna M, Bresnahan M, Bahram S, Strominger JL, Spies T. Allelic variants of the human putative peptide transporter involved in antigen processing. *Proc. Natl. Acad. Sci. U.S.A*. 1992;89(9):3932-3936.

159. Whitton JA, Oldstone MBA. Class I MHC can present an endogenous peptide to cytotoxic T lymphocytes. *J Exp Med*. 1989;170(3):1033-1038.

160. Heemels MT, Ploegh HL. Substrate specificity of allelic variants of the TAP peptide transporter. *Immunity*.

1994;1(9):775-784.

161. Momburg F, Neefjes JJ, Hämmerling GJ. Peptide selection by MHC-encoded TAP transporters. *Curr. Opin. Immunol*. 1994;6(1):32-37.

162. York IA, Rock KL. Antigen processing and presentation by the class I major histocompatibility complex. *Annu. Rev. Immunol*. 1996;14:369-396.

163. Bjorkman PJ, Saper MA, Samraoui B, *et al.* Structure of the human class I histocompatibility antigen, HLA-A2. *Nature*. 1987;329(6139):506-512.

164. Lawlor DA, Zemmour J, Ennis PD, Parham P. Evolution of class-I MHC genes and proteins: from natural selection to thymic selection. *Annu. Rev. Immunol*. 1990;8:23-63.

165. Hughes AL, Nei M. Pattern of nucleotide substitution at major histocompatibility complex class I loci reveals overdominant selection. *Nature*. 1988;335(6186):167-170.

166. Parham P, Ohta T. Population Biology of Antigen Presentation by MHC Class I Molecules. *Science*. 1996;272(5258):67 -74.

167. Bjorkman PJ, Saper MA, Samraoui B, *et al.* The foreign antigen binding site and T cell recognition regions of class I histocompatibility antigens. *Nature*. 1987;329(6139):512-518.

168. Bjorkman PJ, Parham P. Structure, Function, and Diversity of Class I Major Histocompatibility Complex Molecules. *Annu. Rev. Biochem.* 1990;59(1):253-288.

169. Madden DR. The Three-Dimensional Structure of Peptide-MHC Complexes. *Annu. Rev. Immunol.* 1995;13(1):587-622.

170. Saper MA, Bjorkman PJ, Wiley DC. Refined structure of the human histocompatibility antigen HLA-A2 at 2.6 Å resolution. *J. Mol. Biol*. 1991;219(2):277-319.

171. Doherty PC, Zinkernagel RM. A Biological Role fot the Major Histocompatibility Antigens. *The Lancet*. 1975;305(7922):1406-1409.

172. Madden DR, Garboczi DN, Wiley DC. The antigenic identity of peptide-MHC complexes: a comparison of the conformations of five viral peptides presented by HLA-A2. *Cell*. 1993;75(4):693-708.

173. Fremont DH, Matsumura M, Stura EA, Peterson PA, Wilson IA. Crystal structures of two viral peptides in complex with murine MHC class I H-2Kb. *Science*. 1992;257(5072):919-927.

174. Smith MH, Lam KS, Hersh EM, Lebl M, Grimes WJ. Peptide sequences binding to MHC class I proteins. *Mol. Immunol*. 1994;31(18):1431-1437.

175. Johansen TE, McCullough K, Catipovic B, *et al.* Peptide binding to MHC class I is determined by individual pockets in the binding groove. *Scand. J. Immunol*. 1997;46(2):137-146.

176. Yamada N, Ishikawa Y, Dumrese T, *et al.* Role of anchor residues in peptide binding to three HLA-A26 molecules. *Tissue Antigens*. 1999;54(4):325-332.

177. Teng MK, Smolyar A, Tse AG, *et al.* Identification of a common docking topology with substantial variation among different TCR-peptide-MHC complexes. *Curr. Biol*. 1998;8(7):409-412.

178. Garboczi DN, Ghosh P, Utz U, *et al.* Structure of the complex between human T-cell receptor, viral peptide and HLA-A2. *Nature*. 1996;384(6605):134-141.

179. Sami M, Rizkallah PJ, Dunn S, *et al.* Crystal structures of high affinity human T-cell receptors bound to peptide major histocompatibility complex reveal native diagonal binding geometry. *Protein Eng. Des. Sel*. 2007;20(8):397-403.

180. Cella M, Jarrossay D, Facchetti F, *et al.* Plasmacytoid monocytes migrate to inflamed lymph nodes and produce large amounts of type I interferon. *Nat. Med*. 1999;5(8):919-923.

181. Izquierdo-Useros N, Naranjo-Gómez M, Erkizia I, *et al.* HIV and Mature Dendritic Cells: Trojan Exosomes Riding the Trojan Horse? *PLoS Pathog*. 2010;6(3):e1000740.

182. Shattock RJ, Moore JP. Inhibiting sexual transmission of HIV-1 infection. *Nat. Rev. Microbiol*. 2003;1(1):25-34.

183. Turville SG, Arthos J, Donald KM, *et al.* HIV gp120 receptors on human dendritic cells. *Blood*. 2001;98(8):2482-2488.

184. Soto-Ramirez LE, Renjifo B, McLane MF, *et al.* HIV-1 Langerhans' cell tropism associated with heterosexual transmission of HIV. *Science*. 1996;271(5253):1291-1293.

185. Cameron PU, Forsum U, Teppler H, Granelli-Piperno A, Steinman RM. During HIV-1 infection most blood dendritic cells are not productively infected and can induce allogeneic CD4+ T cells clonal expansion. *Clin. Exp. Immunol*. 1992;88(2):226-236.

186. Granelli-Piperno A, Finkel V, Delgado E, Steinman RM. Virus replication begins in dendritic cells during the transmission of HIV-1 from mature dendritic cells to T cells. *Curr. Biol*. 1999;9(1):21-29.

187. Cameron PU, Freudenthal PS, Barker JM, *et al.* Dendritic cells exposed to human immunodeficiency virus type-1 transmit a vigorous cytopathic infection to CD4+ T cells. *Science*. 1992;257(5068):383-387.

188. Pope M, Betjes MG, Romani N, *et al.* Conjugates of dendritic cells and memory T lymphocytes from skin facilitate productive infection with HIV-1. *Cell*. 1994;78(3):389-398.

189. McDonald D, Wu L, Bohks SM, *et al.* Recruitment of HIV and its receptors to dendritic cell-T cell junctions. *Science*. 2003;300(5623):1295-1297.

190. Wiley RD, Gummuluru S. Immature dendritic cell-derived exosomes can mediate HIV-1 trans infection. *Proc. Natl. Acad. Sci. U.S.A*. 2006;103(3):738-743.

191. Kwon DS, Gregorio G, Bitton N, Hendrickson WA, Littman DR. DC-SIGN-mediated internalization of HIV is required for trans-enhancement of T cell infection. *Immunity*. 2002;16(1):135-144.

192. Granelli-Piperno A, Golebiowska A, Trumpfheller C, Siegal FP, Steinman RM. HIV-1-infected monocyte-derived dendritic cells do not undergo maturation but can elicit IL-10 production and T cell regulation. *Proc. Natl. Acad. Sci. U.S.A*. 2004;101(20):7669-7674.

193. Knight SC, Patterson S, Macatonia SE. Stimulatory and suppressive effects of infection of dendritic cells with HIV-1. *Immunol. Lett*. 1991;30(2):213-218.

194. Blagoveshchenskaya AD, Thomas L, Feliciangeli SF, Hung C-H, Thomas G. HIV-1 Nef Downregulates MHC-I by a PACS-1- and PI3K-Regulated ARF6 Endocytic Pathway. *Cell*. 2002;111(6):853-866.

195. Loré K, Sönnerborg A, Broström C, *et al.* Accumulation of DC-SIGN+CD40+ dendritic cells with reduced CD80 and CD86 expression in lymphoid tissue during acute HIV-1 infection. *AIDS*. 2002;16(5):683-692.

196. Messmer D, Jacqué J-M, Santisteban C, *et al.* Endogenously expressed Nef uncouples cytokine and chemokine production from membrane phenotypic maturation in dendritic cells. *J. Immunol*. 2002;169(8):4172-4182.

197. Carrington M, O'Brien SJ. The influence of HLA genotype on AIDS. *Annu. Rev. Med*. 2003;54:535-551.

198. Fellay J, Ge D, Shianna KV, *et al.* Common Genetic Variation and the Control of HIV-1 in Humans. *PLoS Genet*. 2009;5(12):e1000791.

199. Fellay J, Shianna KV, Ge D, *et al.* A Whole-Genome Association Study of Major Determinants for Host Control of HIV-1. *Science*. 2007;317(5840):944 -947.

200. Leslie S, Donnelly P, McVean G. A statistical method for predicting classical HLA alleles from SNP data. *Am. J. Hum. Genet*. 2008;82(1):48-56.

201. Altfeld M, Kalife ET, Qi Y, *et al.* HLA Alleles Associated with Delayed Progression to AIDS Contribute Strongly to the Initial CD8(+) T Cell Response against HIV-1. *PLoS Med*. 2006;3(10):e403.

202. Kosmrlj A, Read EL, Qi Y, *et al.* Effects of thymic selection of the T-cell repertoire on HLA class I-associated control of HIV infection. *Nature*. 2010;465(7296):350-354.

203. Fischer W, Ganusov VV, Giorgi EE, *et al.* Transmission of single HIV-1 genomes and dynamics of early immune escape revealed by ultra-deep sequencing. *PLoS ONE*. 2010;5(8):e12303.

204. Turnbull EL, Lopes AR, Jones NA, *et al.* HIV-1 Epitope-Specific CD8+ T Cell Responses Strongly Associated with Delayed Disease Progression Cross-Recognize Epitope Variants Efficiently. *The Journal of Immunology*. 2006;176(10):6130 -6146.

205. Martinez-Picado J, Prado JG, Fry EE, *et al.* Fitness Cost of Escape Mutations in p24 Gag in Association with Control of Human Immunodeficiency Virus Type 1. *J. Virol.* 2006;80(7):3617-3623.

206. Cao J, McNevin J, Malhotra U, McElrath MJ. Evolution of CD8+ T Cell Immunity and Viral Escape Following Acute HIV-1 Infection. *The Journal of Immunology*. 2003;171(7):3837 -3846.

207. Borrow P, Lewicki H, Wei X, *et al.* Antiviral pressure exerted by HIV-l-specific cytotoxic T lymphocytes (CTLs) during primary infection demonstrated by rapid selection of CTL escape virus. *Nat Med*. 1997;3(2):205-211.

208. Streeck H, Jolin JS, Qi Y, *et al.* Human Immunodeficiency Virus Type 1-Specific CD8+ T-Cell Responses during Primary Infection Are Major Determinants of the Viral Set Point and Loss of CD4+ T Cells. *J. Virol.* 2009;83(15):7641-7648.

209. Champagne P, Ogg GS, King AS, *et al.* Skewed maturation of memory HIV-specific CD8 T lymphocytes. *Nature*. 2001;410(6824):106-111.

210. Harari A, Petitpierre S, Vallelian F, Pantaleo G. Skewed representation of functionally distinct populations of virus-specific CD4 T cells in HIV-1–infected subjects with progressive disease: changes after antiretroviral therapy. *Blood*. 2004;103(3):966 -972.

211. Lichterfeld M, Yu XG, Mui SK, *et al.* Selective depletion of high-avidity human immunodeficiency virus type 1 (HIV-1)-specific CD8+ T cells after early HIV-1 infection. *J. Virol*. 2007;81(8):4199-4214.

212. Streeck H, Brumme ZL, Anastario M, *et al.* Antigen Load and Viral Sequence Diversification Determine the Functional Profile of HIV-1–Specific CD8+ T Cells. *PLoS Med*. 2008;5(5):e100.

213. Miedema F. A brief history of HIV vaccine research: stepping back to the drawing board? *AIDS*. 22(14):1699-1703.

214. Gougeon ML, Lecoeur H, Dulioust A, *et al.* Programmed cell death in peripheral lymphocytes from HIV-infected persons: increased susceptibility to apoptosis of CD4 and CD8 T cells correlates with lymphocyte activation and with disease progression. *J. Immunol*. 1996;156(9):3509-3520.

215. Addo MM, Altfeld M, Rosenberg ES, *et al.* The HIV-1 regulatory proteins Tat and Rev are frequently targeted by cytotoxic T lymphocytes derived from HIV-1-infected individuals. *Proc. Natl. Acad. Sci. U.S.A*. 2001;98(4):1781-1786.

216. Altfeld M, Rosenberg ES, Shankarappa R, *et al.* Cellular immune responses and viral diversity in individuals treated during acute and early HIV-1 infection. *J. Exp. Med*. 2001;193(2):169-180.

217. Rivière Y, McChesney MB, Porrot F, *et al.* Gag-specific cytotoxic responses to HIV type 1 are associated with a decreased risk of progression to AIDS-related complex or AIDS. *AIDS Res. Hum. Retroviruses*. 1995;11(8):903-907.

218. Ndhlovu ZM, Piechocka-Trocha A, Vine S, *et al.* Mosaic HIV-1 Gag Antigens Can Be Processed and Presented to Human HIV-Specific CD8+ T Cells. *The Journal of Immunology*. 2011;186(12):6914 -6924.

219. Barouch DH, O'Brien KL, Simmons NL, *et al.* Mosaic HIV-1 vaccines expand the breadth and depth of cellular immune responses in rhesus monkeys. *Nat Med*. 2010;16(3):319-323.

220. Fischer W, Perkins S, Theiler J, *et al.* Polyvalent vaccines for optimal coverage of potential T-cell epitopes in global HIV-1 variants. *Nat Med*. 2007;13(1):100-106.

221. Goulder PJ, Brander C, Annamalai K, *et al.* Differential narrow focusing of immunodominant human immunodeficiency virus gag-specific cytotoxic T-lymphocyte responses in infected African and caucasoid adults and children. *J. Virol*. 2000;74(12):5679-5690.

222. Frahm N, Kiepiela P, Adams S, *et al.* Control of human immunodeficiency virus replication by cytotoxic T lymphocytes targeting subdominant epitopes. *Nat Immunol*. 2006;7(2):173-178.

223. Gallimore A, Dumrese T, Hengartner H, Zinkernagel RM, Rammensee HG. Protective immunity does not correlate with the hierarchy of virus-specific cytotoxic T cell responses to naturally processed peptides. *J. Exp. Med*. 1998;187(10):1647-1657.

224. Madden DR, Gorga JC, Strominger JL, Wiley DC. The three-dimensional structure of HLA-B27 at 2.1 Å resolution suggests a general mechanism for tight peptide binding to MHC. *Cell*. 1992;70(6):1035-1048.

225. Goulder PJ, Bunce M, Krausa P, *et al.* Novel, cross-restricted, conserved, and immunodominant cytotoxic T lymphocyte epitopes in slow progressors in HIV type 1 infection. *AIDS Res. Hum. Retroviruses*. 1996;12(18):1691-1698.

226. Hoof I, Pérez CL, Buggert M, *et al.* Interdisciplinary analysis of HIV-specific CD8+ T cell responses against variant epitopes reveals restricted TCR promiscuity. *J. Immunol*. 2010;184(9):5383-5391.

227. Mason D. A very high level of crossreactivity is an essential feature of the T-cell receptor. *Immunology Today*. 1998;19(9):395-404.

228. Brander C, Hartman KE, Trocha AK, *et al.* Lack of strong immune selection pressure by the immunodominant, HLA-A*0201-restricted cytotoxic T lymphocyte response in chronic human immunodeficiency virus-1 infection. *J. Clin. Invest*. 1998;101(11):2559-2566.

229. Kan-Mitchell J, Bisikirska B, Wong-Staal F, *et al.* The HIV-1 HLA-A2-SLYNTVATL Is a Help-Independent CTL Epitope. *The Journal of Immunology*. 2004;172(9):5249 -5261.

230. Edwards CTT, Pfafferott KJ, Goulder PJR, Phillips RE, Holmes EC. Intrapatient Escape in the A*0201-Restricted Epitope SLYNTVATL Drives Evolution of Human Immunodeficiency Virus Type 1 at the Population Level. *J Virol*. 2005;79(14):9363-9366.

231. Larsson M, Fonteneau J-F, Lirvall M, *et al.* Activation of HIV-1 specific CD4 and CD8 T cells by human dendritic cells: roles for cross-presentation and non-infectious HIV-1 virus. *AIDS*. 2002;16(10):1319-1329.

232. Buonaguro L, Tornesello ML, Tagliamonte M, *et al.* Baculovirus-Derived Human Immunodeficiency Virus Type 1 Virus-Like Particles Activate Dendritic Cells and Induce Ex Vivo T-Cell Responses. *J Virol*. 2006;80(18):9134-9143.

233. Wagner R, Fliessbach H, Wanner G, *et al.* Studies on processing, particle formation, and immunogenicity of the HIV-1 gag gene product: a possible component of a HIV vaccine. *Arch. Virol*. 1992;127(1-4):117-137.

234. Bachmann MF, Lutz MB, Layton GT, *et al.* Dendritic cells process exogenous viral proteins and virus-like particles for class I presentation to CD8+ cytotoxic T lymphocytes. *Eur. J. Immunol*. 1996;26(11):2595-2600.

235. Ruedl C, Storni T, Lechner F, Bächi T, Bachmann MF. Cross-presentation of virus-like particles by skin-derived CD8(-) dendritic cells: a dispensable role for TAP. *Eur. J. Immunol*. 2002;32(3):818-825.

236. Fausch SC, Da Silva DM, Kast WM. Differential uptake and cross-presentation of human papillomavirus virus-like particles by dendritic cells and Langerhans cells. *Cancer Res*. 2003;63(13):3478-3482.

237. Tsunetsugu-Yokota Y, Morikawa Y, Isogai M, *et al.* Yeast-derived human immunodeficiency virus type 1 p55(gag) virus-like particles activate dendritic cells (DCs) and induce perforin expression in Gag-specific CD8(+) T cells by cross-presentation of DCs. *J. Virol*. 2003;77(19):10250-10259.

238. Yan M, Peng J, Jabbar IA, *et al.* Activation of dendritic cells by human papillomavirus-like particles through TLR4 and NF-kappaB-mediated signalling, moderated by TGF-beta. *Immunol. Cell Biol*. 2005;83(1):83-91.

239. Doan LX, Li M, Chen C, Yao Q. Virus‐like particles as HIV‐1 vaccines. *Reviews in Medical Virology*. 2005;15(2):75-88.

240. Pillay S, Shephard EG, Meyers AE, Williamson A-L, Rybicki EP. HIV-1 sub-type C chimaeric VLPs boost cellular immune responses in mice. *J Immune Based Ther Vaccines*. 2010;8:7.

241. Buonaguro L, Devito C, Tornesello ML, *et al.* DNA-VLP prime-boost intra-nasal immunization induces cellular and humoral anti-HIV-1 systemic and mucosal immunity with cross-clade neutralizing activity. *Vaccine*. 2007;25(32):5968-5977.

242. Bart P-A, Goodall R, Barber T, *et al.* EV01: a phase I trial in healthy HIV negative volunteers to evaluate a clade C HIV vaccine, NYVAC-C undertaken by the EuroVacc Consortium. *Vaccine*. 2008;26(25):3153-3161.

243. Su L, Graf M, Zhang Y, *et al.* Characterization of a Virtually Full-Length Human Immunodeficiency Virus Type 1 Genome of a Prevalent Intersubtype (C/B') Recombinant Strain in China. *J. Virol.* 2000;74(23):11367-11376.

244. Hanahan D. Studies on transformation of Escherichia coli with plasmids. *Journal of Molecular Biology*. 1983;166(4):557-580.

245. Foster DN, Bottoli I, Iacovoni JS, Vogt PK. The DF-1 chicken fibroblast cell line: transformation induced by diverse oncogenes and cell death resulting from infection by avian leukosis viruses. *Virology*. 1998;248(2):295-304.

246. MacPherson I. Polyoma transformation of hamster cell clones--an investigation of genetic factors affecting cell competence. *Virology*. 1962;16:147-151.

247. Gey, GO. Tissue culture studies of the proliferative capacity of cervical carcinoma and normal epithelium. *Cancer Res*. 1952;12(April):264-265.

248. Steel CM, Edmond E. Human lymphoblastoid cell lines. I. Culture methods and examination for Epstein-Barr virus. *J. Natl. Cancer Inst*. 1971;47(6):1193-1201.

249. Graf M, Bojak A, Deml L, *et al.* Concerted action of multiple cis-acting sequences is required for Rev dependence of late human immunodeficiency virus type 1 gene expression. *J. Virol*. 2000;74(22):10822-10826.

250. Dinman JD, Wickner RB. Ribosomal frameshifting efficiency and gag/gag-pol ratio are critical for yeast M1 double-stranded RNA virus propagation. *J Virol*. 1992;66(6):3669-3676.

251. Saiki RK, Gelfand DH, Stoffel S, *et al.* Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science*. 1988;239(4839):487-491.

252. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem*. 1976;72:248-254.

253. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*. 1970;227(5259):680-685.

254. Lanier LL, Warner NL. Paraformaldehyde fixation of hematopoietic cells for quantitative flow cytometry (FACS) analysis. *J. Immunol. Methods*. 1981;47(1):25-30.

255. Thurner B, Röder C, Dieckmann D, *et al.* Generation of large numbers of fully mature and stable dendritic cells from leukapheresis products for clinical application. *Journal of Immunological Methods*. 1999;223(1):1-15.

256. Bredl S, Wagner R. Modulation der Immunogenität einer auf Gag, Pol und Nef basierenden HIV-1 Vakzine. 2009.

257. Zagury JF, Cantalloube H, Achour A, *et al.* Striking similarities between HIV-1 Env protein and the apoptosis mediating cell surface antigen Fas. Role in the pathogenesis of AIDS. *Biomedecine & Pharmacotherapy*. 1993;47(8):331-335.

258. Chen C-J, Kono H, Golenbock D, *et al.* Identification of a key pathway required for the sterile inflammatory response triggered by dying cells. *Nat. Med.* 2007;13(7):851-856.

259. Gheysen D, Jacobs E, de Foresta F, *et al.* Assembly and release of HIV-1 precursor Pr55gag virus-like particles from recombinant baculovirus-infected insect cells. *Cell*. 1989;59(1):103-112.

260. Shastri N, Serwold T, Gonzalez F. Presentation of endogenous peptide/MHC class I complexes is profoundly influenced by specific C-terminal flanking residues. *J. Immunol*. 1995;155(9):4339-4346.

261. Ahr, Barbara, Robert-Hebmann, Vernoique, Devaux, Christian, Biard-Piechaczyk, Martine. Apoptosis of uninfected cells induced by HIV envelope glycoproteins. *Retrovirology*. 2004;1:12, 2004.

262. Demain AL, Vaishnav P. Production of recombinant proteins by microbes and higher organisms. *Biotechnology Advances*. Mai;27(3):297-306.

263. Schubert U, Antón LC, Gibbs J, *et al.* Rapid degradation of a large fraction of newly synthesized proteins by proteasomes. *Nature*. 2000;404(6779):770-774.

264. Strack B, Calistri A, Craig S, Popova E, Göttlinger HG. AIP1/ALIX Is a Binding Partner for HIV-1 p6 and EIAV p9 Functioning in Virus Budding. *Cell*. 2003;114(6):689-699.

265. Fujii K, Hurley JH, Freed EO. Beyond Tsg101: the role of Alix in „ESCRTing" HIV-1. *Nat Rev Micro*. 2007;5(12):912-916.

266. Vogel FR. The role of adjuvants in retroviral vaccines. *Int. J. Immunopharmacol.* 1995;17(2):85-90.

267. Gupta RK, Siber GR. Adjuvants for human vaccines--current status, problems and future prospects. *Vaccine*. 1995;13(14):1263-1276.

268. Gupta RK, Relyveld EH, Lindblad EB, *et al.* Adjuvants--a balance between toxicity and adjuvanticity. *Vaccine*. 1993;11(3):293-306.

269. Mahnke K, Schmitt E, Bonifaz L, Enk AH, Jonuleit H. Immature, but not inactive: the tolerogenic function of immature dendritic cells. *Immunol Cell Biol*. 2002;80(5):477-483.

270. Hisamatsu H, Shimbara N, Saito Y, *et al.* Newly identified pair of proteasomal subunits regulated reciprocally by interferon gamma. *The Journal of Experimental Medicine*. 1996;183(4):1807 -1816.

271. Harding CV, Unanue ER. Quantitation of antigen-presenting cell MHC class II/peptide complexes necessary for T-cell stimulation. *Nature*. 1990;346(6284):574-576.

272. Christinck ER, Luscher MA, Barber BH, Williams DB. Peptide binding to class I MHC on living cells and quantitation of complexes required for CTL lysis. *Nature*. 1991;352(6330):67-70.

273. Pang KC, Wei JQZ, Chen W. Dynamic quantification of MHC class I-peptide presentation to CD8+ T cells via intracellular cytokine staining. *Journal of Immunological Methods*. 2006;311(1-2):12-18.

274. Pardigon N, Bercovici N, Calbo S, *et al.* Role of co-stimulation in CD8+ T cell activation. *Int. Immunol.* 1998;10(5):619-630.

275. Ojcius DM, Abastado JP, Casrouge A, *et al.* Dissociation of the peptide-MHC class I complex limits the binding rate of exogenous peptide. *J. Immunol.* 1993;151(11):6020-6026.

276. Sijts AJ, Pamer EG. Enhanced intracellular dissociation of major histocompatibility complex class I-associated peptides: a mechanism for optimizing the spectrum of cell surface-presented cytotoxic T lymphocyte epitopes. *J. Exp. Med.* 1997;185(8):1403-1411.

277. Faries MB, Bedrosian I, Reynolds C, *et al.* Active macromolecule uptake by lymph node antigen-presenting cells: a novel mechanism in determining sentinel lymph node status. *Ann. Surg. Oncol.* 2000;7(2):98-105.

278. Allan RS, Waithman J, Bedoui S, *et al.* Migratory Dendritic Cells Transfer Antigen to a Lymph Node-Resident Dendritic Cell Population for Efficient CTL Priming. *Immunity*. 2006;25(1):153-162.

279. Qian S-B, Reits E, *et al.* Tight Linkage between Translation and MHC Class I Peptide Ligand Generation Implies Specialized Antigen Processing for Defective Ribosomal Products. *J Immunol*. 2006;177(1):227 -233.

280. Princiotta MF, Finzi D, Qian S-B, *et al.* Quantitating protein synthesis, degradation, and endogenous antigen processing. *Immunity*. 2003;18(3):343-354.

281. Grant E, Michalek M, Goldberg A, Rock K. Rate of antigen degradation by the ubiquitin-proteasome pathway influences MHC class I presentation. *The Journal of Immunology*. 1995;155(8):3750 -3758.

282. Townsend A, Bastin J, Gould K, *et al.* Defective presentation to class I-restricted cytotoxic T lymphocytes in vaccinia-infected cells is overcome by enhanced degradation of antigen. *The Journal of Experimental Medicine*. 1988;168(4):1211 -1224.

283. Tobery T, Siliciano RF. Cutting Edge: Induction of Enhanced CTL-Dependent Protective Immunity In Vivo by N-End Rule Targeting of a Model Tumor Antigen. *The Journal of Immunology*. 1999;162(2):639 -642.

284. Kubota H. Quality Control Against Misfolded Proteins in the Cytosol: A Network for Cell Survival. *Journal of Biochemistry*. 2009;146(5):609 -616.

285. Cyr DM, Hebert DN. Protein quality control - linking the unfolded protein response to disease. *EMBO Rep*. 2009;10(11):1206-1210.

286. Heck JW, Cheung SK, Hampton RY. Cytoplasmic protein quality control degradation mediated by parallel actions of the E3 ubiquitin ligases Ubr1 and San1. *Proceedings of the National Academy of Sciences*. 2009

287. Schubert U, Antón LC, Gibbs J, *et al.* Rapid degradation of a large fraction of newly synthesized proteins by proteasomes. *Nature*. 2000;404(6779):770-774.

288. Hartl FU, Bracher A, Hayer-Hartl M. Molecular chaperones in protein folding and proteostasis. *Nature*. 2011;475(7356):324-332.

289. Goldwich A, Hahn SSC, Schreiber S, *et al.* Targeting HIV-1 Gag into the Defective Ribosomal Product Pathway Enhances MHC Class I Antigen Presentation and CD8+ T Cell Activation. *The Journal of Immunology*. 2008;180(1):372 -382.

290. de Felipe P, Hughes LE, Ryan MD, Brown JD. Co-translational, intraribosomal cleavage of polypeptides by the foot-and-mouth disease virus 2A peptide. *J. Biol. Chem*. 2003;278(13):11441-11448.

291. Rivett AJ. Proteasomes: multicatalytic proteinase complexes. *Biochem. J*. 1993;291 (Pt 1):1-10.

292. Rechsteiner M, Hoffman L, Dubiel W. The multicatalytic and 26 S proteases. *J. Biol. Chem*. 1993;268(9):6065-6068.

293. Wong SBJ, Buck CB, Shen X, Siliciano RF. An Evaluation of Enforced Rapid Proteasomal Degradation as a Means of Enhancing Vaccine-Induced CTL Responses. *The Journal of Immunology*. 2004;173(5):3073 -3083.

294. Toapanta FR, Craigo JK, Montelaro RC, Ross TM. Reduction of anti-HIV-1 Gag immune responses during co-immunization: immune interference by the HIV-1 envelope. *Curr. HIV Res.* 2007;5(2):199-209.

295. Loré K, Betts MR, Brenchley JM, *et al.* Toll-like receptor ligands modulate dendritic cells to augment cytomegalovirus- and HIV-1-specific T cell responses. *J. Immunol.* 2003;171(8):4320-4328.

296. Williams MA, Trout R, Spector SA. HIV-1 gp120 Modulates the Immunological Function and Expression of Accessory and Co-Stimulatory Molecules of Monocyte-derived Dendritic Cells. *Journal of Hematotherapy Stem Cell Research*. 2002;11:829-847.

297. Capobianchi MR. Induction of lymphomonocyte activation by HIV-1 glycoprotein gp120. Possible role in AIDS pathogenesis. *Journal of biological regulators and homeostatic agents*. 10(4):83-91.

298. Datta SK, Raz E. Induction of antigen cross-presentation by Toll-like receptors. *Springer Semin. Immunopathol.* 2005;26(3):247-255.

299. Mouriès J, Moron G, Schlecht G, *et al.* Plasmacytoid dendritic cells efficiently cross-prime naive T cells in vivo after TLR activation. *Blood*. 2008;112(9):3713-3722.

300. Tritel M, Resh MD. Kinetic Analysis of Human Immunodeficiency Virus Type 1 Assembly Reveals the Presence of Sequential Intermediates. *J. Virol.* 2000;74(13):5845-5855.

301. Chazal N, Carrière C, Gay B, Boulanger P. Phenotypic characterization of insertion mutants of the human immunodeficiency virus type 1 Gag precursor expressed in recombinant baculovirus-infected cells. *J Virol*. 1994;68(1):111-122.

302. Wagner R, Deml L, Schirmbeck R, Reimann J, Wolf HJ. Induction of a MHC class I-restricted, CD8 positive cytolytic T-cell response by chimeric HIV-1 virus-like particles in vivo: implications on HIV vaccine development. 1994.

303. Buonaguro L, Tornesello M, Lewis G, Buonaguro F. Structured particles for antigen presentation. *Retrovirology*. 3(Suppl 1):S28-S28.

304. Vassilieva EV, Wang B-Z, Vzorov AN, *et al.* Enhanced Mucosal Immune Responses to HIV Virus-Like Particles Containing a Membrane-Anchored Adjuvant. *mBio*. January;2(1).

305. Aricò E, Wang E, Tornesello ML, *et al.* Immature monocyte derived dendritic cells gene expression profile in response to Virus-Like Particles stimulation. *J Transl Med*. 3:45-45.

306. Smith CL, Mirza F, Pasquetto V, *et al.* Immunodominance of Poxviral-Specific CTL in a Human Trial of Recombinant-Modified Vaccinia Ankara. *The Journal of Immunology*. 2005;175(12):8431 -8437.

307. Ustrell V, Realini C, Pratt G, Rechsteiner M. Human lymphoblast and erythrocyte multicatalytic proteases: differential peptidase activities and responses to the 11S regulator. *FEBS Letters*. 1995;376(3):155-158.

308. Bellone M, Iezzi G, Manfredi AA, *et al.* In vitro priming of cytotoxic T lymphocytes against poorly immunogenic epitopes by engineered antigen-presenting cells. *Eur. J. Immunol.* 1994;24(11):2691-2698.

309. Santegoets SJAM, Schreurs MWJ, Masterson AJ, *et al.* In vitro priming of tumor-specific cytotoxic T lymphocytes using allogeneic dendritic cells derived from the human MUTZ-3 cell line. *Cancer Immunol. Immunother.* 2006;55(12):1480-1490.

310. Zhang S, Zhang H, Zhao J. The role of CD4 T cell help for CD8 CTL activation. *Biochemical and Biophysical Research Communications*. 2009;384(4):405-408.

311. O A. *The Chicago Manual of Style. 16. ed.* 2010. Aufl. Chicago: The University of Chicago

312. McNaught A. *Compendium of Chemical Terminology: IUPAC Recommendations. 2. ed*. Oxford, England; Malden MA, USA: Blackwell Science; 1997.

313.OA.*Journal of Biological Chemistry: Abbreviations* Available from: http://www.jbc.org/site/misc/abbrev.xhtml